

Global Coagulation Assays in Haemophilia:

Comparison and Correlation with Conventional Assays

and Clinical Phenotype

by

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A portfolio of research and development in a professional context

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**THESIS CONTAINS
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Abstract

Background: The global assays assess the interaction of procoagulants and anticoagulants on the generation of thrombin. These assays, as functional tests, may reflect the bleeding tendency in patients with haemophilia better than the conventional assays.

Design and Methods: In this study the use of three global assays including rotational thromboelastometry (ROTEM[®]), thrombin generation test (TGT), and clot waveform analysis were evaluated on a cohort of haemophilia A (HA) and haemophilia B (HB) patients. The global assay parameters were compared to the conventional assays and the most sensitive parameters correlated with the clinical phenotype of haemophilia patients.

Results and discussion: The modified ROTEM[®] analysis initiated by a minute amount of tissue factor proved to be useful in assessing haemophilia patients. A strong correlation was found between the Maximum Velocity (MaxVel) parameter of ROTEM[®] and the factor VIII level of haemophilia individuals. The use of corn trypsin inhibitor (CTI) to inhibit contact pathway improved the sensitivity and specificity of the ROTEM[®] analysis. A significant difference between the TGT parameters of patients groups and the control was noted. The use of CTI improved the sensitivity and specificity of the test, in particular peak height in platelet rich plasma (PRP) samples. The results in PRP+CTI group showed that platelets play an important role in heterogeneity of thrombin generation amongst severe haemophilia patients where the relevant deficient factor is < 1.0 IU/dL compared to conventional clotting assays.

In clot waveform analysis, this study showed a better correlation between the velocity indices of the APTT test (Min1 and Min2) and the FVIII activity of HA individuals compared to the correlation between the APTT test itself and the FVIII activity level. The Min1 and Min2 appear to be more sensitive, simple, fast and cost effective in the diagnosis of hypocoagulability, and monitoring of haemophilia patients compared to the conventional tests.

The area under the thrombin generation curve (AUC), peak height (PH), and time to peak (TP) parameters of the in-house TGT in PRP+CTI test category showed a strong correlation with patient age at first joint bleed ($r = 0.693$ $p = 0.003$, $r = 0.718$ $p = 0.002$, and $r = -0.703$ $p = 0.002$ respectively). The type of *F8* mutation was also correlated with the patient's thrombin generation capacity. Based on two subgroups of null mutations and non-null mutations, the PH and AUC in PPP+CTI category showed a significant increase in the non-null mutation group. The time to the maximum velocity of ROTEM[®] (tMaxVel) was also shorter in the non-null mutation group.

Conclusion: The use of global assays in diagnosing hypocoagulability, and monitoring haemophilia patients proved to be useful, especially in areas where conventional methods are not responsive. However, the multifactorial dependency of global assays make them difficult to interpret on their own, therefore, these assays should be used in parallel with the conventional tests in order to be more useful.

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Abbreviations

α 2-M	α 2-Macroglobulin
AA	arachidonic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ALT	alanine aminotransferase
APC	activated protein C
APTT	activated partial thromboplastin time
AST	aspartate aminotransferase
AT	antithrombin
AUC	area under the curve
bp	base pair
C4BP	complement 4 binding protein
CAT	calibrated automated thrombogram
CFT	clot formation time
CRM	cross reacting materials
CT	clot time
CTI	corn trypsin inhibitor
CV	coefficient of variation
CWB	citrated whole blood
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ETP	endogenous thrombin potential
<i>F8</i>	factor VIII gene
<i>F9</i>	factor IX gene
FBC	full blood count
FII	factor II
FIX	factor IX

FV	factor V
FVII	factor VII
FVIII	factor VIII
FVL	factor V Leiden
FX	factor X
FXI	factor XI
FXII	factor XII
FXIII	factor XIII
γ -GT	γ -glutamyl transferase
GLA	glutamic acid
GP	glycoprotein
HA	haemophilia A
HB	haemophilia B
HEPES	hydroxyethy piperazineethanesulfonic acid
HSBT	high salt wash buffer with tween
HWB	HEPES working buffer
ICH	International conference on harmonisation
kb	kilo base
KDHCTU	Katharine Dormandy Haemophilia Centre & Thrombosis unit
LI	lysis index
MaxVel	maximum velocity
MCF	maximum clot firmness
Min1	minimum value for first derivative
Min2	minimum value for second derivative
ML	maximum lysis
mRNA	messenger ribonucleic acid
OPD	orthophenylene-diamine
PAI	plasminogen activator inhibitor
PAR	protease activated receptor
PC	protein C
PCR	polymerase chain reaction
PEG	polyethylene glycol
PH	peak height

PL	phospholipid
PL:PC	phospholipid: phosphatidyl choline
PL:PE	phospholipid: phosphatidyl ethanolamine
PL:PS	phospholipid: phosphatidyl serine
pNA	para-nitroanilide
PPP	platelet poor plasma
PRP	platelet rich plasma
PS	protein S
PT	prothrombin time
PT3'	prothombin 3' untranslated region mutation
rFVII	recombinant factor VII
rFVIII	recombinant factor VIII
rTF	recombinant tissue factor
RFU	relative fluorescence unit
ROC	receiver operating characteristic
ROTEM [®]	rotational thromboelastometry
SD	standard deviation
TAFI	thrombin activatable fibrinolysis inhibitor
TEG	thrombelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGT	thrombin generation test
TM	thrombomodulin
tMaxVel	time to maximum velocity
tPA	Tissue-type plasminogen activator
TT	thrombin time
uPA	urokinase plasminogen activator
VTE	venous thromboembolism
VWF	von Willebrand factor
VWF:CB	von Willebrand factor: collagen binding
WHO	world health organisation

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Declaration

I declare that whilst studying for the Doctorate in Biomedical Science at the University of Portsmouth I have not been registered for any other award at another university. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained within this submission is my own and, the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgment has been made in the text.

Saman Aghighi

May 2011

Dedication

To my wife Zohreh and my son Leo

Publications

Abstracts

Aghighi S., Riddell A.F., Chowdary P., Gatt A. (2009). Comparison of three different thrombin generation assays in patients with haemophilia. *Poster presentation at the International Society on Thrombosis and Haemostasis.*

Aghighi S., Riddell A.F., Chowdary P., Lilley P., Griffioen A., Tuddenham E. G.(2007). Thrombin generation test (TGT) and rotational thromboelastometry (ROTEM[®]) in assessing severe haemophilia A phenotype. *Poster presentation at the International Society on Thrombosis and Haemostasis.*

Chapter 1 Introduction

Maintenance of blood flow within the vascular system is an important human physiological process. The term 'haemostasis' refers to the process of cessation of the bleed from the injured vessel by forming a clot that serves to limit haemorrhage. Thrombosis, however, is the pathological clot formation that results when haemostasis is excessively activated in the absence of bleeding, and/or inadequate response by anticoagulant factors to regulate procoagulant activities.

Under normal physiological conditions there is a delicate equilibrium between the pathological states of hypercoagulability and hypocoagulability in the circulating blood.

Haemostasis involves:

- Vasoconstriction of damaged blood vessels to decrease blood flow within the vessel.
- Platelets and their interaction with the disrupted vessel and other components of the coagulation system.
- The formation of fibrin by the coagulation system.
- The regulation of the extension of the blood clot by coagulation inhibitors and the fibrinolytic system.
- The remodelling and repair of the injured vessel.

1.1. Overview of normal haemostasis

When a blood vessel is damaged, the first physiologic reaction is vasoconstriction that helps to slow the blood flow. The disrupted endothelial cell barrier exposes collagen, which attracts platelets, anuclear cells that participate in many aspects of clot formation from the circulating blood and initiate the first adhesion of platelets to the exposed collagen, followed by aggregation of platelets, which is mediated by von Willebrand factor (VWF). This forms a primary haemostatic plug, preventing further blood flow loss from the injured vessel. The primary plug also provides a surface for the blood coagulation process.

The disrupted vessel wall also exposes tissue factor (TF), a membrane protein located in smooth muscle, fibroblasts, and macrophages that combines with circulating coagulation factor VIIa (FVIIa) to trigger the coagulation system leading to a stepwise activation of a series of proenzymes to produce thrombin. In turn, thrombin converts fibrinogen to insoluble fibrin that is then cross-linked by the action of factor XIIIa (FXIIIa), and anchored into place by the process of clot retraction. The complexity of coagulation results from numerous feedback loops and inhibitory mechanisms that balance coagulation and fibrinolysis.

1.1.1. Blood vessels

Blood vessels are lined with a continuous layer of endothelial cells that protect the underlying basal membrane, subendothelium and smooth muscle, and their highly reactive elements from contacting blood cells, platelets, and coagulation proteins. These elements include adhesive proteins such as collagen, fibronectin, laminin,

vitronectin, and VWF, which promote platelet adhesion, and TF that triggers blood coagulation.

The degree of platelet adherence to an injured vessel wall is dependent upon the relative reactivity of the components of the vessel wall that have become exposed. Slight trauma or irritation to the endothelium will result in reversible activation of platelets and limited activation of the clotting system. Injury to, or detachment of the endothelium, will expose the basal membrane and/or subendothelium to blood. This interaction and simultaneous expression of TF (by exposure) will fully and irreversibly activate platelets and the coagulation system.

Vasoconstriction of the damaged vessel occurs in response to the localised release of vasoactive amines from activated platelets. Damaged vessel walls also mediate the activation of fibrinolysis, kinin formation and the complement system (Hoffbrand *et al.*, 1999). There are several coagulation inhibitors that are produced by endothelial cells. Endothelial cells express heparan sulphate, a glycosaminoglycan, which catalyses anticoagulant activity of antithrombin (AT). Plasma AT binds to heparan sulphate located on the luminal surface, and in the basement membrane of the endothelium. Thrombomodulin is another endothelium-bound protein with anticoagulant function, facilitating activation of protein C (PC) and thrombin activatable fibrinolysis inhibitor (TAFI). In response to systemic procoagulant stimuli, tissue-type plasminogen activator (tPA) is transiently released from endothelial cells to promote fibrinolysis. Endothelium activated by inflammation modulates procoagulant responses by synthesising TF, VWF, and plasminogen activator inhibitor (PAI-1) (Tanaka *et al.*, 2009).

1.1.2. Platelets

Blood platelets are fragments of the cytoplasm of the megakaryocyte, and hence are anuclear and formed in the bone marrow. The megakaryocytes are derived from pluripotential stem cells and their precursors are megakaryoblasts. Thrombopoietin, a glycoprotein hormone which is produced by liver and kidney, is the major regulator of platelet production (Kato *et al.*, 1998). It stimulates the production and differentiation of megakaryocytes. Each megakaryocyte may give rise to as many as four thousand platelets (Hoffbrand *et al.*, 2006).

Primary haemostasis is triggered in response to vessel wall damage by the exposure of blood to subendothelial tissue. Platelet membrane is vital to the process because of the specific glycoprotein (GP) receptors, through which platelets interact with other components of haemostasis (Kaushansky, 1995). Before the first interaction of a platelet with the injured vessel wall, VWF binds to exposed collagens in the vessel wall. The initial contact of platelets can then take place via the interaction of the platelet receptor complex GPIb/V/IX with the immobilised VWF. This interaction initiates the tethering of circulating platelets to the vessel wall. Platelets roll over VWF in the direction of flow driven by the shear forces. The adhesion of platelets to subendothelium involves the functioning of at least four other receptors: the collagen receptors $\alpha_2\beta_1$ (GPIa-IIa) and GPVI, the fibronectin receptor $\alpha_5\beta_1$ and the fibrinogen receptor $\alpha_{IIb}\beta_3$ (GPIIb-IIIa). Spreading of the platelet will follow the firm adhesion and is essential to withstand the shear forces exerted by the flowing blood (Gresle, 2002). Once platelets are bound to the vessel wall they can be activated by a variety of ligands, such as interaction of GPVI with collagen, and activation by thrombin. Thrombin activates platelets through protease-activated receptors (PAR). There are

four PARs (designated PAR1-4), of which, only PAR1 and PAR4 are found on human platelets. It is thought that PAR1 is necessary for platelet response at low thrombin concentration (Kahn *et al.*, 1999). Platelets next undergo a shape change by becoming more spherical with extended pseudopods thus spreading over the exposed subendothelium (Bouaziz *et al.*, 2007). The contents of the platelet granules are released: α -granules release fibrinogen, VWF, thrombospondin, factor V (FV), vitronectin and other proteins; the dense bodies release adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, and calcium. Cytoplasmic events include activation of the eicosanoid pathway (Begonja *et al.*, 2007), decreased cAMP and increased mobilisation of Ca^{2+} (Calvete, 1999).

As platelets become activated, they become adhesive for other platelets interacting via fibrinogen bound to their GPIIb-IIIa receptors. The activation of other platelets occurs by release and secretion of thromboxane A_2 , ADP, Ca^{2+} , and serotonin from activated platelet's granules. Thromboxane A_2 is the major product of membrane arachidonic acid (AA) metabolism. During activation, under control of a transport mediating protein called "scramblase", negatively charged phospholipid (PL), phosphatidylserine (PL:PS) are translocated to the outer surface of the plasma membrane. In addition, microvesiculation of the membrane occurs. The microvesicles further enhance surface expression of PL:PS, which acts as a binding surface for activated forms of factor V (FVa), factor IX (FIXa), and factor X (FXa), which in the presence of Ca^{2+} form the prothrombinase complex (see below) (Calvete, 1999). The formation of the primary platelet plug temporarily occludes the site of damage to prevent blood loss.

1.1.3. Coagulation

The activated platelets stimulate local activation of plasma coagulation factors by the presentation of negatively charged phospholipids on their surface. Activation of coagulation factor receptors and release of α granules content (i.e. FV, VWF and fibrinogen) set the scene for activation of coagulation factors. The small quantities of thrombin (0.05 to 1.00 nM), resulting from the activation of the TF pathway are sufficient to activate platelet protease-activated receptors (PAR) and FXI on the platelet surface in the presence of calcium ions.

Coagulation factors are glycosylated proteins that circulate in the blood as inert proenzymes. Once stimulated, the proenzymes become active, triggering a process of serial cascade activation. There are two main groups of coagulation factors that can be categorised by their modes of action. The first, zymogens (inactive precursor of an enzyme) factors II, VII, IX, X, XI, XII and prekallikrein, become serine protease enzymes upon activation (factors IIa, VIIa, IXa, Xa, XIa, XIIa and kallikrein). Their active site is a serine residue with nearby histidyl groups and they can work in two different ways. Firstly, there is limited proteolysis and this enzymatic reaction reveals the active site of the target protein through partial cleavage. Secondly, there is a conformational change revealing the active site without any disintegration of the target molecule. Of the serine proteases, factors IIa, VIIa, IXa and Xa form have a common feature, which is the presence of carboxylated glutamyl acid residues that enable binding to phospholipids. The last stage of their production, γ -carboxylation, is a post-translational vitamin K-dependent step (Davie *et al.*, 1991).

The second group of factors are co-factors (factors Va and VIIIa), which circulate as pre-cofactors factor V (FV) and factor VIII (FVIII), and do not take part directly in any proteolytic event but are necessary for acceleration of the pathway. They enhance the activity of target proteins by increasing local concentrations of coagulation factors on phospholipid surfaces, creating a condition favourable for proteolysis.

In 1964, two independent groups of scientists introduced a model of coagulation as a series of reactions in which one clotting factor activates the next one leading to fibrin clot formation. One group proposed the “cascade” model (Macfarlane, 1964), and this was shortly followed by the second group suggesting the “waterfall” model (Davie & Ratnoff, 1964). The cascade or waterfall models described each clotting factor as a proenzyme that could be converted to an active enzyme. These models also suggested that the clotting sequences were divided by two pathways: the intrinsic pathway and the extrinsic pathway. The initiation of either pathway resulted in activation of a common pathway and the eventual generation of fibrin clot (Luchtman-Jones & Broze, 1995). (Figure 1.1)

The limitations of the coagulation cascade as a model of the haemostatic process are highlighted by certain clinical observations. Patients deficient in the initial components of the intrinsic pathway (FXII, high-molecular weight kininogen, or prekallikrein) have a prolonged activated partial thromboplastin time but no bleeding tendency. In spite of this fact, components of the intrinsic pathway must have an important role in haemostasis, because patients deficient in FVIII or factor IX (FIX) have a serious bleeding tendency, although the extrinsic pathway is intact.

Coagulation Cascade

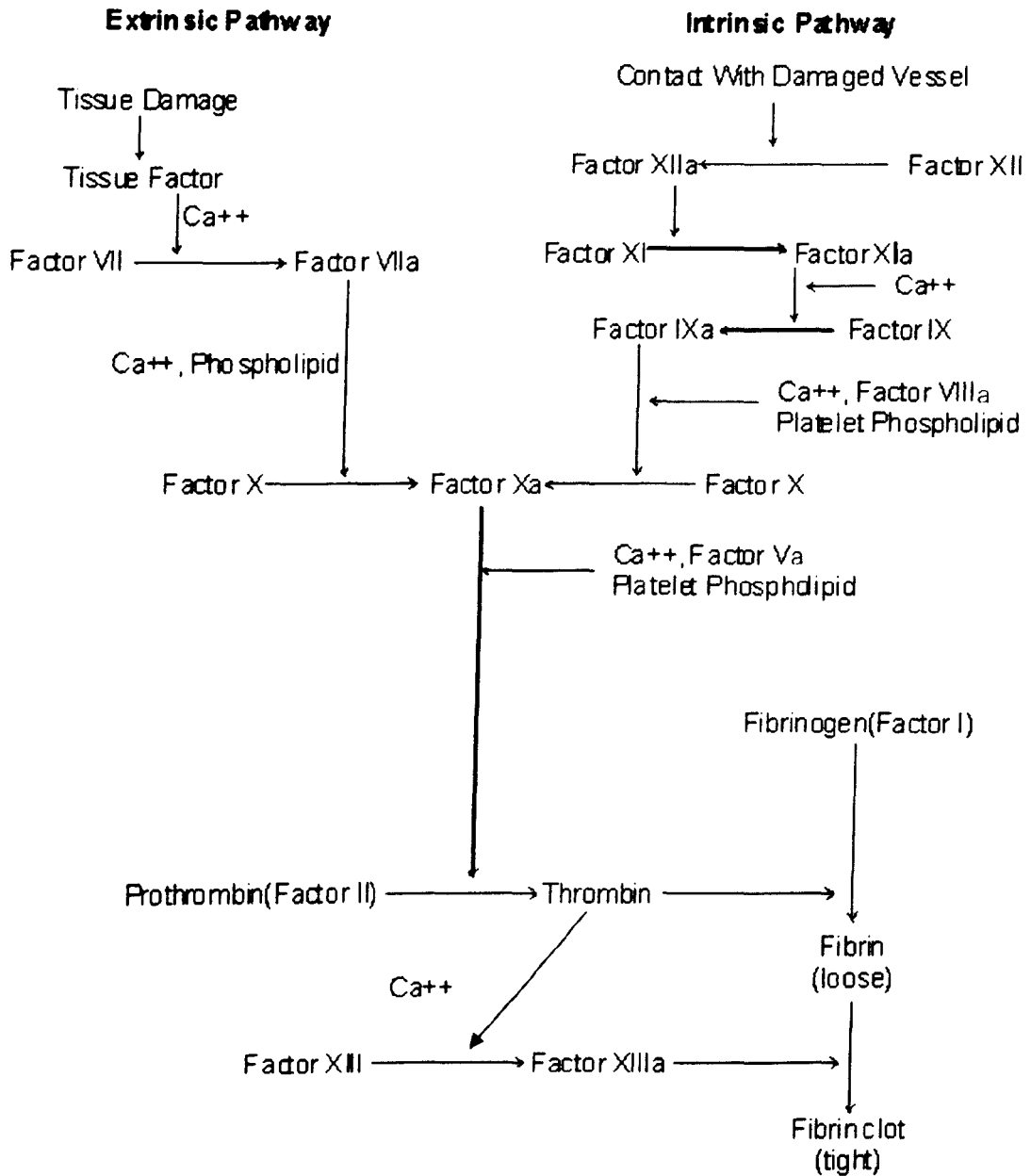


Figure 1.1 The Coagulation cascade.

The Coagulation cascade/waterfall first described in 1964 (Davie & Ratnoff, 1964; Macfarlane, 1964).

Similarly, patients deficient in FVII also have a serious bleeding tendency, even though the intrinsic pathway is intact. Thus, the intrinsic and extrinsic pathways cannot operate as independent pathways *in vivo* as they do in the cascade model.

Other observations showed that patients with FXI deficiency, although not usually subject to spontaneous haemorrhage, were prone to excessive bleeding after surgery or trauma (Hu *et al.*, 1998). These observations pointed to the pivotal roles of FVIIIa and FIXa in the activation of FX, and made particularly interesting the observation that FIX activation could be catalysed not only by FXIa, but also by FVIIa-TF, suggesting that the TF pathway could initiate the intrinsic pathway directly and independently of the contact factor pathway. However, this did not explain the haemostatic defect observed in FXI deficiency, which therefore suggested that the activation of FXI physiologically might proceed by a pathway independent of contact activation pathway (Walsh, 2004). An alternative mechanism for proteolytic activation of FXI by thrombin, demonstrated in 1991, was subsequently shown to proceed at 5000- to 10,000-fold accelerated rates on the activated platelet surface (Naito & Fujikawa, 1991).

The cascade model of coagulation also does not explain the amplification of thrombin by its own positive feedback process by activating FV, FVIII, and platelets (Butenas *et al.* 1997) (see 1.1.4.2).

1.1.4. Cell-based model of coagulation

A major development over the recent years was the discovery that exposure of blood to cells that express TF on their surface, such as epithelial cells, is both necessary and

sufficient to initiate blood coagulation *in vivo*. This finding led to the belief that the intrinsic pathway (the contact system) does not have a true physiological role in haemostasis (Hoffman & Monroe, 2001). Mounting evidence suggests that TF is present in blood on cellular microparticles. These membrane fragments derive from various cell types: white blood cells, endothelium, and platelets, which may play a more important role in pathological haemostasis (thrombosis) as opposed to normal clotting (Osterud & Bjorklid, 2006). In the cell-based model of coagulation, coagulation occurs in three overlapping phases: initiation, priming (amplification), and propagation (Figure 1.2) (Monroe *et al.*, 2002b).

1.1.4.1. Initiation phase

The blood coagulation reactions are initiated when sub-endothelial TF is exposed to the blood flow following damage or injury. The initiation of coagulation may also occur as a consequence of activation of endothelium by chemicals, cytokines, or inflammatory processes (Butenas & Mann, 2007). The initiation phase of coagulation occurs when exposed TF (expressed on fibroblasts, monocytes and macrophages) binds to circulating FVIIa that exists in very small quantities (approximately 1 % of FVII) (Mann *et al.*, 1998). TF is the high-affinity cellular receptor for coagulation FVIIa (Butenas *et al.*, 2005) and in the absence of TF, FVIIa has very low catalytic activity, therefore, binding to TF is necessary to render FVIIa functional by an allosteric mechanism. The TF-FVIIa complex activates FIX and factor X (FX) to the activated forms FIXa and FXa, of which the latter, initially, is the more efficient substrate. Therefore, the formation of TF-FVIIa-FXa is termed the extrinsic Xase complex (Nemerson, 1988). The amount of FXa is limited as its generation is rapidly down-regulated by tissue factor pathway inhibitor (TFPI). This small amount of FXa

activates FV to FVa on the surface of TF bearing cell, and together they generate a small amount (picomolar concentrations) of thrombin (Monroe *et al.*, 2002).

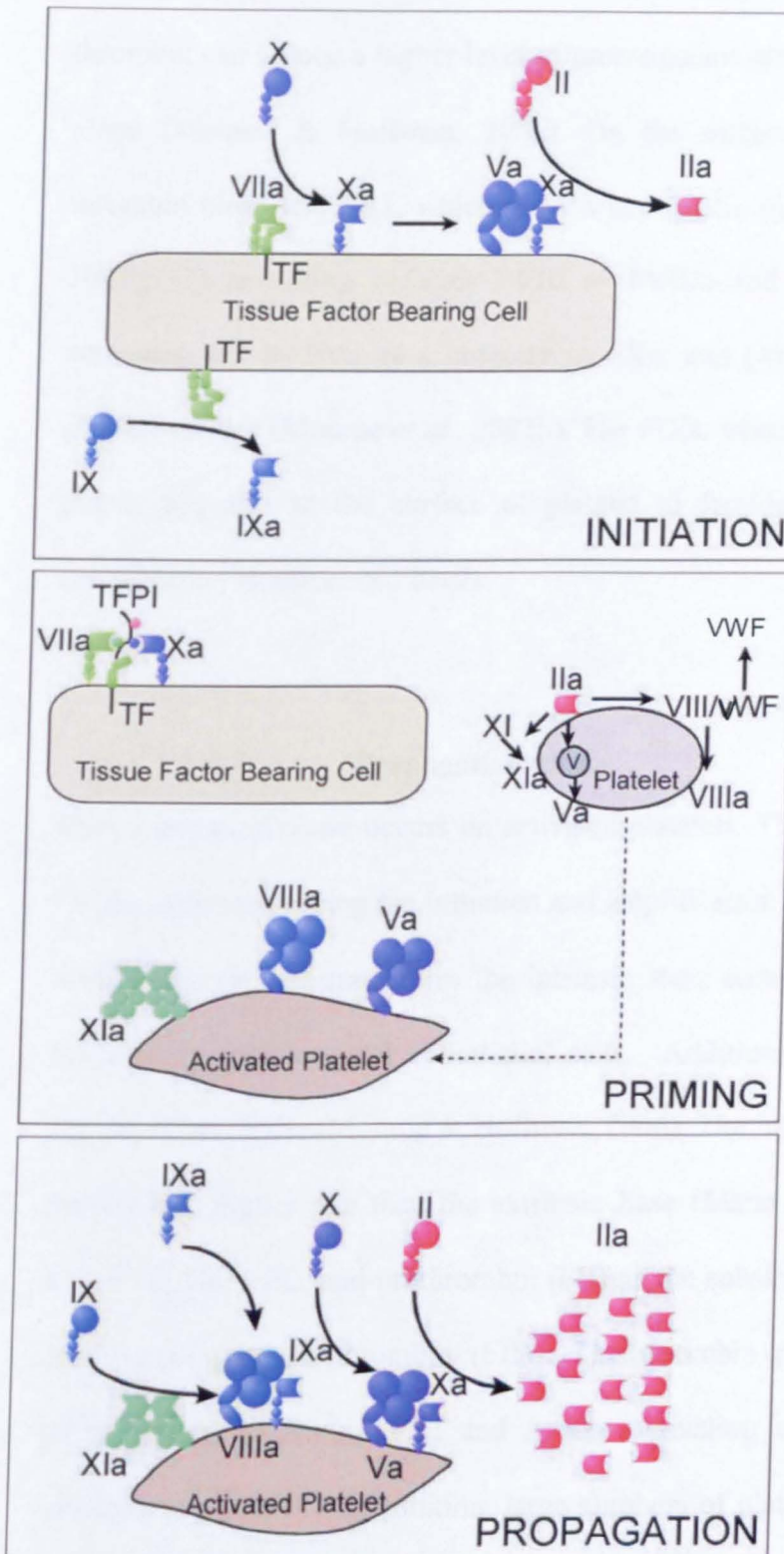


Figure 1.2 Cell-based model of coagulation:

Initiation:

In the initiation phase, factor VIIa bound to TF activates factor IX and also factor X. Factor Xa then activates factor V on the TF-bearing cell, complexes with factor Va, and converts a small amount of FII to FIIa.

Priming:

The small amount of initial thrombin activates platelets, causing release of α granule contents including FV. The released FV, then activated by the thrombin. Also thrombin activates FXI and FVIII by cleaving it from VWF.

Propagation:

FIXa generated by factor VIIa/TF binds to the activated platelets and subsequently activates FX. This FIXa is supplemented by FIXa generated on the platelet surface by FXIa. FXa together with FVa forms prothrombinase complex, resulting in a burst of thrombin (adapted from (Monroe *et al.*, 2002b), with permission).

1.1.4.2. Amplification (priming) phase

In the amplification (priming) phase, the small amount of thrombin produced on TF-bearing cells activates cofactors FV and FVIII, and platelets. Although platelets have already adhered at the site of injury and become partially activated, the addition of thrombin can induce a higher level of procoagulant activity than adhesive interactions alone (Monroe & Hoffman, 2006). On the surface of platelets membrane, (1) thrombin binds to PAR1, which plays a key role in platelet activation (Smyth *et al.*, 2007); (2) activating cofactor FVIII to FVIIIa and releasing it from VWF; (3) activating FV to FVa as a cofactor to FXa; and (4) activating FXI bound to the platelet surface (Monroe *et al.*, 2002b). The FIXa, which is generated during initiation phase, migrates to the surface of platelet to facilitate the propagation phase of coagulation (Moore *et al.*, 2010).

1.1.4.3. Propagation phase

The propagation phase occurs on activated platelets. The enzyme FIXa, the cofactor FVIIIa, activated during the initiation and amplification phases, respectively, together with FX as the substrate form the intrinsic Xase complex on a membrane surface provided by platelets and endothelial cells. Additional FIXa is also activated by platelet-bound FXIa (Monroe & Hoffman, 2006). The intrinsic Xase activates FX at a 50-100 fold higher rate than the extrinsic Xase (Mann *et al.*, 1998). FXa, together with FVa, Ca^{2+} , PL, and prothrombin (FII) as the substrate form the prothrombinase complex to produce thrombin (FIIa). The thrombin produced amplifies its own generation by activating FXI and further activation of platelets, FV and FVIII (Butenas *et al.*, 1997). In addition, large numbers of platelets can be recruited to the site of injury to provide enough surfaces for large-scale thrombin generation.

Thrombin cleaves fibrinopeptides A and B from fibrinogen, to expose recognition sites that polymerise the fibrinogen into fibrin strands. Fibrin acts as a template to accelerate thrombin activation of thrombin activatable fibrinolysis inhibitor (TAFI) to protect the clot against proteolysis. Also, thrombin cleaves factor XIII (FXIII) to form the insoluble cross-linked fibrin clot (Monroe & Hoffman, 2006). Factor XIII also binds to α_2 -antiplasmin, the main inhibitor of plasmin, which is responsible for dissolution of the clot (Moore *et al.*, 2010).

In most models of coagulation, the formation of the fibrin clot occurs at the start of the propagation phase when the burst of platelet surface thrombin generation is just beginning. Consequently, more than 95% of total thrombin production takes place after initial clot formation. It has been proposed that the excess thrombin is produced to activate the TAFI, thereby protecting the clot against proteolysis (Mann *et al.*, 2003a). TAFI is a carboxypeptidase that removes terminal lysine residues from fibrin, thereby, removing potential binding sites for fibrinolytic enzymes and enhancing clot resistance to fibrinolysis. Failure of TAFI activation is thought to contribute significantly to the bleeding tendency in haemophilia (Mosnier *et al.*, 2001).

1.1.5. Regulation of coagulation

Three major groups of inhibitors control and regulate coagulation activity. These are serine protease inhibitors, the activated co-factor inhibitors, and the extrinsic pathway inhibitor. They are all produced principally in the liver and their action is triggered before the process of fibrin deposition has commenced (Hoffbrand *et al.*, 2006).

1.1.5.1. Serine protease inhibitors (serpins)

The serine protease inhibitors are glycoproteins that inhibit the activated procoagulants. The best known serine protease inhibitor is Antithrombin (AT) (Hoffman & Monroe, 2007). AT is a single chain glycoprotein, which is synthesised principally in the liver and inhibits the activity of thrombin and FXa, and to a lesser degree other serine proteases, such as FIXa, FXIa, and FVIIa in the FVIIa-TF complex , but not free FVIIa (Moore *et al.*, 2010).

AT inactivates its substrate by binding irreversibly through an arginine residue to the active serine site of the protease by forming a 1:1 complex with the enzyme at their active site, which prevents the procoagulant from participation in the coagulation process. The rate of inactivation is relatively slow, but activity of AT is greatly enhanced by the anticoagulant heparin, or *in-vivo*, by heparan sulphate, a proteoglycan located on the surface of endothelial cells. They produce a conformational change in AT as a result of which the reactive arginine residue becomes more readily able to combine with and inhibit the serine active centre of thrombin and other serine protease clotting factors. Heparin induces a 2300-fold increase in the rate of thrombin inactivation by AT such that its action becomes almost instantaneous. Congenital deficiencies of AT often lead to recurrent thrombosis in adult life (Rezaie, 2002).

Other serpins include heparin co-factor II (which is a selective thrombin inhibitor), C₁ inhibitor (which neutralises kallikrein, factors XIIa and XIa) and α_2 -macroglobulin (which inhibits thrombin and kallikrein) (Hoffman & Monroe, 2007).

1.1.5.2. Activated cofactors inhibitors

The main activated co-factor (FVa and FVIIIa) inhibitor is activated protein C (APC) (Hepner *et al.*, 2002). Protein C is a vitamin K-dependent glycoprotein, synthesised in the liver. It circulates as an inactive zymogen, which is converted to an active serine protease, APC, by the proteolytic action of thrombin. Activation is accelerated by an endothelial surface protein, thrombomodulin (TM), through attachment of PC to endothelial protein C receptor (EPCR), which forms a complex with thrombin simultaneously inhibiting the procoagulant activity of thrombin on fibrinogen, FV, FVIII and platelets. APC cleaves and inactivates FVa and VIIIa so that they can no longer interact with FXa and FIXa respectively. The inhibitory action of APC occurs on phospholipid surfaces.

Protein S (PS), a non-enzymatic APC co-factor, specifically enhances the inhibitory activity of APC, by promoting its binding to the platelet membrane phospholipid and orienting it above the surface of activated platelet (Moore *et al.*, 2010). PS is also a vitamin K-dependent glycoprotein synthesised in the liver and endothelial cells. About 40% of the PS in plasma is in the free form, whilst the remaining 60% is associated in a 1:1 complex with the C₄b-binding protein (C₄BP) and does not enhance PC function.

1.1.5.3. Tissue factor pathway inhibitor (TFPI)

The inhibitors mentioned above cause little or no inhibition of FVIIa. Instead the action of FVIIa is modulated by a specific extrinsic pathway inhibitor known as tissue factor pathway inhibitor (TFPI). TFPI has no effect on FVIIa alone but acts on FVIIa-TF complex, in the presence of factor Xa, in which the two proteases are inactivated sequentially. Most of the TFPI is associated with, and thought to be synthesised by,

endothelial cells, with a smaller amount being found in platelets and plasma (Monroe & Key, 2007).

1.1.6. Fibrinolysis

The fibrinolytic system (see Figure 1.3) exists to restore normal blood flow by restricting the extent of clot formation and dissolving the formed fibrin clot. The main protein involved in this process is plasminogen. It is present in plasma mainly adsorbed onto fibrinogen and fibrin molecules. Plasminogen can be activated to form plasmin, directly by tissue plasminogen activator (tPA) or urokinase or by proteolytic enzymes such as trypsin, factor XIIa, thrombin and kallikrein (Mosnier *et al.*, 2001).

Fibrinogen lysis begins with plasmin cleavage of polypeptides from the carboxyl-terminal (C-terminal) part of lysine on the A α chains, followed by the removal of the first 42 amino acids from N-terminal end of B β chains of fibrinogen. This large residual portion is termed fragment X, which is asymmetrically degraded further to release one fragment D, in which the chains remain linked by disulphide bonds. The remainder of the parent molecule is termed fragment Y, which is again cleaved by plasmin, removing a second fragment D and leaving the disulphide-linked N-terminal ends of all six chains, which are referred as fragment E (Hoffbrand *et al.*, 1999). Cross-linked fibrin is cleaved by plasmin in a similar way to fibrinogen; however, because of the presence of cross links, the resultant products are D-dimer, D-dimer-E fragments, and oligomers of fragment X and Y, in addition to X, Y, D, and E. Plasmin promotes its own generation in two ways. On one hand, continuous cleavage of fibrin by plasmin generates new C-terminal lysine residues that act as binding sites for plasminogen and tPA.

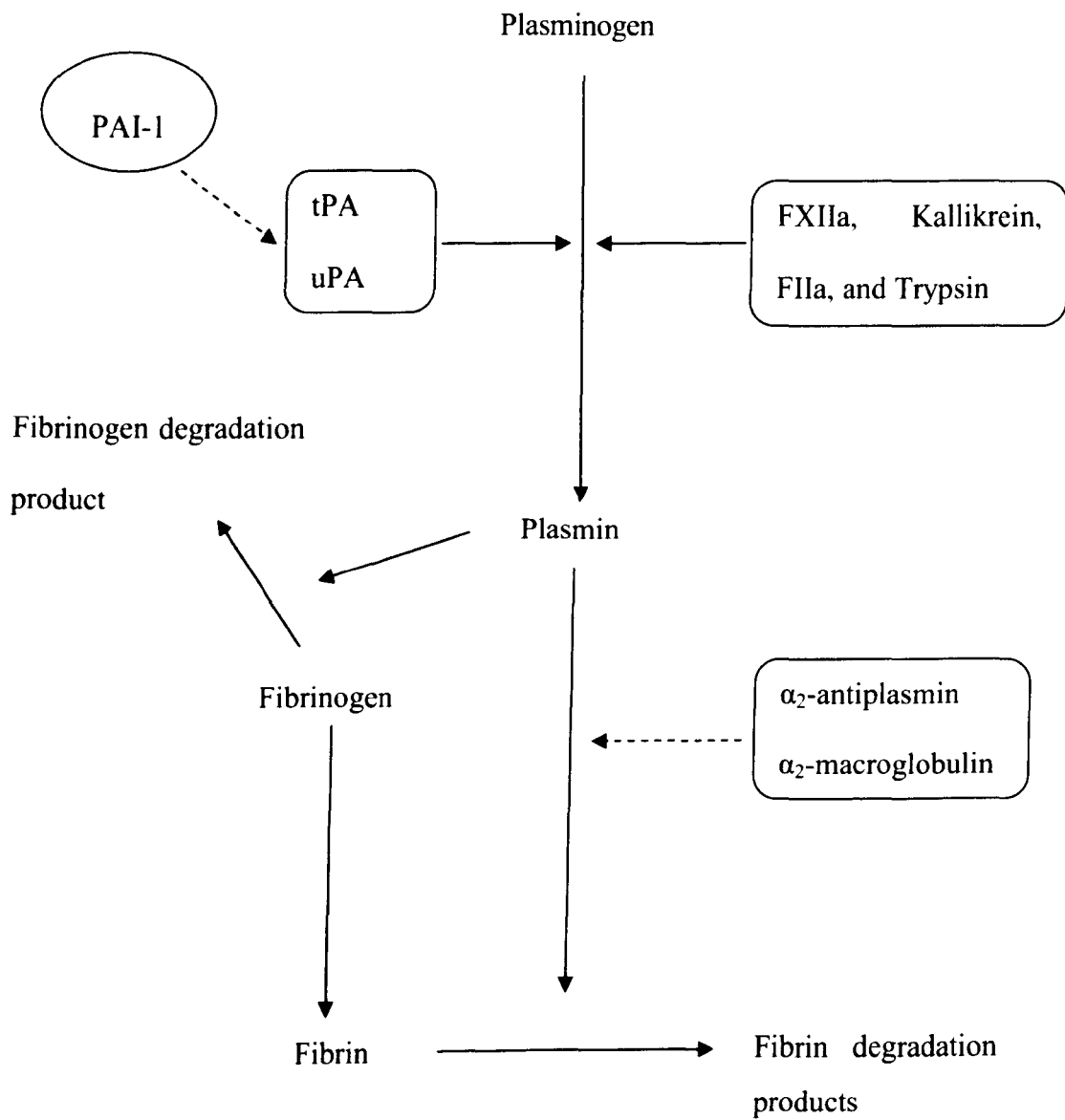


Figure 1.3 The fibrinolysis system
(Broken arrows denote inhibition)

On the other hand, plasmin promotes its own formation by proteolytically removing Glu-plasminogen to generate Lys-plasminogen. Lys-plasminogen is a better substrate for tPA (Mosnier *et al.*, 2001).

1.1.6.1. Inhibitors of fibrinolysis

The fibrinolytic system is also controlled by two main types of inhibitors. Firstly, those that inhibit plasmin, the main one being α_2 -antiplasmin, a glycoprotein that binds to the active site of plasmin, effectively inactivating it. The other group inhibit the activation of plasminogen and include plasminogen activator inhibitor (PAI types 1, 2, and 3) (Lee *et al.*, 2000). PAI-1 and PAI-2 are fast acting inhibitors of tPA. PAI-1 is secreted into the circulation from endothelial cells, whereas PAI-2 is mainly synthesised in the placenta and is only present in blood in high concentrations in mid-late pregnancy. PAI-3 is an inhibitor of tPA, urokinase, thrombin and APC. It is present in blood and its action can be stimulated by heparin.

1.1.6.2. Inhibition of fibrinolysis by TAFI

During the initiation phase of fibrinolysis, limited amounts of plasmin are generated. These small amounts of plasmin are not sufficient to degrade the fibrin clot but enable fibrinolysis to progress into its propagation phase. Plasmin promotes transition of fibrinolysis into the propagation phase by truncating Glu-plasminogen into Lys-plasminogen and by limited proteolysis of fibrin, thereby creating fibrin with C-terminal lysine residues. TAFI inhibits fibrinolysis by inactivating plasmin-mediated auto-feedback loops that are designed to generate a burst of plasmin formation. The C-terminal lysine residues of fibrin are removed by TAFI (Mosnier & Bouma, 2006).

1.2. Haemophilia

Haemophilia is a bleeding disorder resulting from a congenital deficiency of FVIII (Haemophilia A), FIX (Haemophilia B) or FXI (Haemophilia C). It was first described in the Talmud, a collection of Jewish Rabbinical writings from the 2nd century AD, which stated that circumcision should not occur if two siblings had died from bleeding (Rosner, 1969). In the 12th century, the Arab physician, Albucasis, wrote of a family whose males died of bleeding after minor injuries. Dr. John Conrad Otto has been credited with writing the first recent account of hemophilia in his 1803 dissertation, *An Account of a Hemorrhagic Disposition Existing in Certain Families*. However, the word “Haemophilia” did not appear until 1823 when a student at the University of Zurich in Germany used the term (Giangrande, 2004).

Haemophilia first achieved extensive attention when it became known as “the royal disease.” Queen Victoria was a carrier of haemophilia, probably as a result of a spontaneous mutation. Two of her daughters were carriers of haemophilia, and one of her sons, Leopold, had haemophilia and died at the age of thirty-one. Alexandra, a carrier of the disease and a granddaughter of Queen Victoria, married Czar Nicholas II of Russia and had a haemophilic son, Alexis, in 1904. Alexandra sought the help of Rasputin for her son, which enabled Rasputin to gain power in the government and indirectly cause the overthrow of the Romanov Dynasty (Stevens, 1999). Recently their type haemophilia was identified by applying genomic methodologies (multiplex target amplification and massively parallel sequencing) to historical specimens from the Romanov branch of the royal family. A mutation was found in FIX gene on the X chromosome, which caused severe haemophilia B (Rogaev *et al.*, 2009).

In 1944 researchers found in one case that when the blood from two different individuals with haemophilia was mixed, both were able to clot. Nobody could explain this finding until 1952, when researchers in England realized there were two types of haemophilia. They had been studying a 10-year-old boy with haemophilia named Stephen Christmas who did not seem to have the "typical" disease. They called his version haemophilia B, also called "Christmas disease," and the more prevalent kind haemophilia A (HA), also called "classic haemophilia"(Aggeler *et al.*, 1952).

Factor XI deficiency (Haemophilia C) was first described in 1953 in a Jewish family in the United States by Rosenthal *et al.* Two sisters had abnormal bleeding after a tooth extraction and surgery to remove tonsils. Four more of the thirteen members of the family over four generations were identified as factor XI deficient. (It was not in the scope of this thesis to study haemophilia C patients)

1.2.1. Haemophilia A

Haemophilia A (HA), the most common hereditary coagulation disorder, affects 1 in 5000 male births worldwide (Mannucci, 2002). HA is an X-linked recessive disorder which is caused by a qualitative or quantitative abnormality of plasma FVIII protein. However, about 30 percent of cases arise from a spontaneous mutation, where there is no family history of haemophilia (Mannucci & Tuddenham, 2001). Females can be affected if both X chromosomes inherit the defective gene. Another mechanism for HA in females has been described: the presence of two *de novo* factor VIII mutations, an X chromosome deletion, and a paternal FVIII inversion mutation (Windsor *et al.*, 1995). In some haemophilia carriers, as a result of extreme "lyonisation", the X

chromosome carrying the normal genes (FVIII/FIX) is inactivated randomly. This may result in low FVIII/FIX level and bleeding phenotype (Ingerslev *et al.*, 1989).

1.2.1.1. Molecular genetics of haemophilia A

The human FVIII gene (*F8*) was cloned between 1982 and 1984 by Gitschier and collaborators and, at the same time, by Toole *et al.* (Lee *et al.*, 2005; Toole *et al.*, 1992). The *F8* is located at the most distal band, Xq28, of the long arm of the X chromosome. The gene contains 26 exons, nearly 186 kilobases (kb). The 25 intervening regions (introns) that separate the exons range in size from 207 to 32400 base pairs (bp). The FVIII messenger RNA (mRNA) encodes a 2351-aminoacid polypeptide and is synthesised in the liver, spleen, and lymph nodes. Upon secretion, a 19-aminoacid N-terminal leader sequence is cleaved (Hoyer, 1994). The mature FVIII plasma protein of 2332-amino acids contains a domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2 (Figure 1.4). The secreted FVIII is consists of a heavy chain (A1-A2-B) linked non-covalently to a light chain (A3-C1-C2). FVIII circulates in plasma complexed non-covalently with VWF that acts as a plasma carrier, and protects it from proteolysis and rapid clearance (Bhopale & Nanda, 2003).

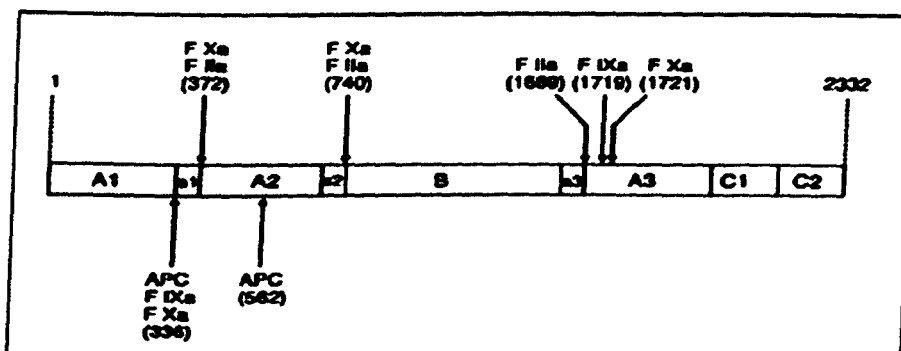


Figure 1.4 Factor VIII peptide.

Factor VIII protein depicted with its domain structure with specific cleavage sites for thrombin, FXa and APC; and binding sites for interaction with FIXa.

a = activated factor; APC = activated protein C; F = factor. Adapted from (Pruthi, 2005; Pruthi & Nichols, 1999).

HA is classified as mild, moderate or severe, based on the factor level in plasma and the severity of clinical disease. Approximately 40-50% of patients with a mutant *F8* have severe haemophilia (the level of FVIII activity is < 1% of normal) whereas the remainder have moderate or mild haemophilia (FVIII activity, 1- 5% of normal or > 5-40% of normal respectively) (Lozier & Kessler, 2000).

HA is caused by a broad spectrum of mutations that can occur along the entire length of the *F8*. The mutations lead to defects at the level of transcription, translation, or to change of individual amino acids in the FVIII protein. The online HA mutation database (<http://europium.csc.mrc.ac.uk>) lists over 2000 individual reports of FVIII variations from all over the world.

F8 defects can be divided into several categories:

- i. Gross gene rearrangements of DNA sequence (inversions);
- ii. Deletions of genetic sequence of a size varying from one base-pair up to the entire gene;
- iii. Single DNA base substitutions, resulting in either amino acid replacement (missense), premature peptide chain termination (non-sense or stop mutation), or mRNA splicing defects;
- iv. Insertion of DNA of varying size.

Inversion gene defects usually involve intron 22 of the *F8*, which causes severe HA and is found in 40%–50% of patients with severe disease. The *F8* is completely disrupted: introns 1 to 22 are moved away from their normal context and their orientation is inverted. The intron 22 inversion is principally an error of DNA

replication during spermatogenesis in males. The distal inversion is more common than the proximal inversion (Tuddenham *et al.*, 1991).

The second most common mutation in severe HA is the intron 1 inversion mutation. This mutation is present in approximately 1.8% of severe HA patients in the UK (Keeney *et al.*, 2005).

Point mutations are the most prevalent type of defect, probably underlying the disease in 90–95% of patients. Point mutations are comprised of missense mutations, nonsense point mutations, and mRNA splice-site point mutations (Bowen, 2002). Point mutations leading to new stop codons are essentially all associated with a severe phenotype, as are most frame-shift mutations. An exception is the insertion or deletion of adenosine bases resulting in a sequence of eight to ten adenosines, which may result in moderately severe HA (Nakaya *et al.*, 2001). Splice site mutations are often severe but may be mild, depending on the specific change and location. Missense mutations occur in fewer than 20% of individuals with severe HA but nearly all of those with mild or moderately severe bleeding tendencies (Keeney *et al.*, 2005). Deletions, the second most common gene defects (5–10% of patients), and insertions are extremely rare.

HA patients are distinguished by the presence or absence of circulating FVIII protein and are divided into two main groups, termed cross-reacting material-negative (CRM⁻) and -positive (CRM⁺), with different molecular pathogenesis. CRM⁻ haemophilia is characterized by a gross reduction or absence of circulating FVIII antigen (FVIII:Ag) as a result of altered folding, secretion or stability in plasma:

FVIII activity (FVIII coagulant (FVIII:C)) is reduced in parallel with FVIII:Ag. CRM⁺ haemophilia is characterized by dysfunctional protein circulating at essentially normal FVIII:Ag levels (but with lower/absent FVIII:C) in which the mutation affects FVIII function but not secretion or stability. A subgroup of CRM⁺, CRM-reduced (CRM^r), is defined by mildly reduced circulating FVIII:Ag, together with further reduced FVIII:C, the mutation affecting both function and secretion or stability (David *et al.*, 2001).

Amino acid substitutions cause the majority of moderate and mild forms of HA. The mechanisms, by which missense mutations lead to decreased FVIII activity in plasma, depend on the localization and nature of substituted amino-acid residues. Such mutations have been described in domains A1, A2, A3 and C2. They are usually associated with the moderate or mild CRM⁻ HA (Gale *et al.*, 2000).

Certain amino acid substitutions in the region a3 and domains C1 and C2 may become an obstacle to the normal binding of FVIII to VWF. The result is shortening of the mutant FVIII plasma half-life and mild or moderate CRM⁻ HA. This can be exemplified by a substitution His2155Asp, which is associated with a change of surface charge of the C1 domain and marked reduction of FVIII binding to VWF. Another example is a substitution of sulphated Tyr1680 in the region a3, which points to the importance of post-translation modification for functional interactions of FVIII. The 3D model revealed that in the C2 domain mutations are localized in the region involved in binding both VWF and the phospholipid membrane (Gilbert *et al.*, 2002). Two types of mutations lead to insufficient thrombin activation of FVIII. The consequence is usually moderate or mild, CRM⁺ haemophilia with dysfunctional

FVIII in plasma. One type of mutation alters amino acids within the thrombin cleavage sites. In addition, substitutions outside the cleavage sites have been described, e. g. substitution of sulphated Tyr349 in domain A1 (Michnick *et al.*, 1994).

A group of missense mutations involves amino acids forming the interfaces between domains A1 and A2 and domains A2 and A3. Substitutions of these residues cause destabilization of the non-covalent interaction of the A2 domain within heterotrimer of the activated FVIII and thus lead to premature termination of its activity. In such patients with mild or moderate haemophilia, lower plasma FVIII activity is detected when determined using the two-stage assay as compared to the one stage assay (Pipe *et al.*, 2001).

Interaction between FVIIIa and FIXa on the surface of the phospholipid membrane can be disturbed by missense mutations in the A3 domain that serves as the receptor site for FIXa. Amino acid substitutions within the A2 domain may affect, on the one hand residues that are in direct contact with the catalytic domain of FIXa and, on the other hand, the more distant residues, where an effect on the conformation of the contact surface can be presumed. The consequence of these mutations is a decrease of FVIIIa cofactor activity resulting in CRM⁺ haemophilia of severity ranging from severe to mild (Jenkins *et al.*, 2002).

1.2.1.2. The role of FVIII in coagulation

Upon secretion into plasma, FVIII is bound to VWF in non-covalent form. FVIII cannot be activated until released from VWF. The role of VWF is to localise FVIII at the site of vascular injury through binding to subendothelial matrix protein and

adherent platelets. On exposure to thrombin or FXa, a proteolytic cleavage at Arg 740 transiently converts the heterogeneous heavy chains into 92-kd fragments (Bhopale & Nanda, 2003). At the same time, a small fragment is cleaved from the light chain to separate FVIII from VWF. Cleavage at Arg1689 appears to be the mechanism by which VWF disassociates from FVIII. These cleavages produce the activated FVIII heterotrimer factor VIIIa (FVIIIa), thereby relieving the inhibitory activity of VWF on FVIII, permitting the FVIIIa to interact with negatively charged PL. Concurrently, the B domain of FVIII is also removed (Hoyer, 1994).

The cofactor activity of FVIIIa in the assembled Xase complex is provided by three essential interactions: binding to the PL membrane, the enzyme FIXa, and the substrate FX. FVIIIa increases the catalytic activity of FIXa by several orders of magnitude on a PL surface. This results in approximately 50-fold more efficient conversion of FX into FXa. Thus, the role of FVIIIa is to generate large amounts of FXa and in this way to provide efficient propagation of the clotting process, and consequently, a burst of thrombin generation (Fay, 2004).

1.2.2. Haemophilia B

Haemophilia B is caused by defects in, or absence of, coagulation FIX. Haemophilia B is a recessively inherited X-linked bleeding disorder, which is less common than HA with prevalence of 1 in 25000 male births worldwide (Mitchell *et al.*, 2005).

1.2.2.1. Molecular genetics of haemophilia B

The FIX gene (*F9*) was cloned in 1982 by three independent groups (Green *et al.*, 1993). It is smaller and structurally simpler than the *F8*, and located near the terminus of the long arm of the X chromosome (Xq27.1). It is approximately 34 kb in length and contains only eight exons and seven introns (Figure 1.5). The number of exons and splice junction types are highly conserved in homologous vitamin K-dependent proteins, e.g. FX, FVII, and protein C (Green *et al.*, 1993).

The first exon encodes the prepeptide (also called signal or leader sequence). The prepeptide is cleaved off, following secretion, by a prepeptidase (Lee *et al.*, 2005). The prepeptide is followed by another signalling peptide, the propeptide, which is encoded in exon b. This 18-residue sequence is the signal for modification, post-translationally, at 12 glutamic acid residues by a vitamin K-dependent gamma-carboxylase to produce γ -carboxyglutamic acid. This is called glutamic acid-rich (GLA) domain region (Handford *et al.*, 1991). The GLA is followed by 2 epidermal growth factor (EGF) domains, an activation peptide, and a catalytic domain (Figure 1.5) (Pruthi, 2005).

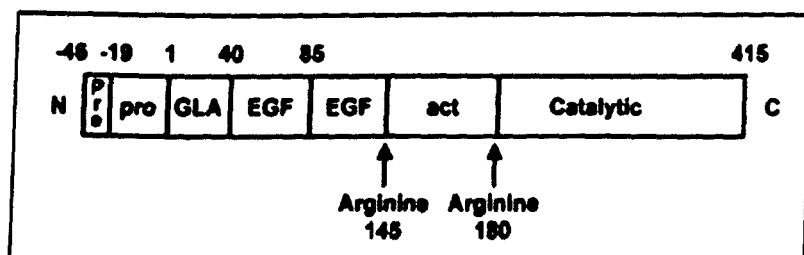


Figure 1.5 Factor IX peptide.

FIX is activated by FXIa or FVIIa-TF complex in a sequential cleavage at Arg 145 and 180 residues. act=activation peptide; C=carboxyl terminus; EGF=epidermal growth factor; GLA=glutamic acid rich; N=amino terminus; Pre=prepeptide; pro=propeptide.(Pruthi, 2005)

Haemophilia B is a mutationally heterogeneous disorder. Most mutations are single base pair changes that result in missense, frame-shift, or nonsense mutations. Short deletions (less than 30 nucleotides) account for approximately 7%, larger deletions approximately 3%, and insertions approximately 2% of mutations. Many of the single base pair changes occur at CpG doublets that are “hot spots” for mutation (Green *et al.*, 1993). “Coldspots” also exist in the gene, where missense mutations are rare or absent. These correlate with parts of the protein that are not required for catalytic activity and are cleaved off during activation (Giannelli *et al.*, 1993).

Missense mutations account for most mutations that typically result in mild disease unless the mutations occur in residues critical for normal FIX function. Some mutations in the promoter region of the *F9* result in a unique phenotype, termed haemophilia B Leyden, which is characterised by severe disease at birth with progressive amelioration of severity throughout adolescence and puberty. The post-pubertal increase in FIX activity in haemophilia B Leyden patients has been suggested to be caused by androgens. This idea was supported by the observation that danazol, an androgen derivative, stimulates FIX activity in pre-pubescent haemophilia B Leyden patients (Boland *et al.*, 1995).

However, some promoter mutations (e.g. Brandenburg mutation at bp 26) in the FIX promoter result in a severe lifelong disease state (Morgan *et al.*, 1997). Nonsense mutations in the signal peptide and propeptide regions lead to severe haemophilia B.

Mutations in the GLA domain disrupt γ -carboxylation (post-translational modification) and calcium binding, the latter is important for normal FIXa binding to collagen, activated platelets, and endothelial cells. Mutations in the EGF domains

disrupt protein-protein interaction (FVIIIa and FVIIa-tissue factor), resulting in reduced activation of FIX. Also, EGF mutations affect binding to Ca^{2+} (Giannelli *et al.*, 1993).

An unusual FIX variant due to mutation at Ala-10 is characterised by normal baseline FIX activity. However, Warfarin therapy results in a severe and disproportionate reduction in FIX activity (typically < 1%), which causes bleeding in patients being treated with Warfarin. An indication of such a situation is a disproportionate prolongation of the activated partial thromboplastin time (APTT), which should prompt clotting factor assays (Pruthi, 2005).

1.2.2.2. The role of FIX in coagulation

Factor IX participates in two physiologically important reactions: cleavage of specific peptide bonds to generate the activated form, FIXa, and cleavage of a specific substrate, FX, by the FIXa. In the first reaction, either FVIIa-TF or FXIa can cleave FIX to generate FIXa. In the second reaction, FIXa in the presence of cofactor FVIIIa, Ca^{2+} , and PL cleaves FX to generate FXa (High & Roberts, 1995).

1.2.3. Clinical phenotype of haemophilia

Haemophilias A and B are clinically indistinguishable from each other. Diagnosis must be confirmed by specific factor assay. Patients with haemophilia display a spectrum of bleeding manifestations (deep muscle and joint haemorrhage, haematomas, easy bruising, post traumatic bleeding, bleeding post-oral injury and tooth extraction, etc). The propensity to musculoskeletal haemorrhage can lead to

recurrent haemarthroses (target joints) and chronic muscle injury and fibrosis (Goodnight *et al.*, 2001).

The bleeding tendency is related to the measured concentration of the factor and is classified as mild, moderate, or severe (Table 1.1). This classification generally predicts bleeding risk, guides the optimum management strategy, and predicts outcome (White *et al.*, 2001). The number of bleeding episodes, the amount of factor used and the presence of arthropathies reflect the course of the disease in each patient (Tizzano *et al.*, 2002a). Although most patients with severe haemophilia need regular replacement therapy, a small proportion of individuals with severe haemophilia exhibit a milder clinical presentation and rarely bleed and need only occasional treatment.

Table 1.1 Classification of haemophilia

Concentration of FVIII/FIX	Classification	Clinical manifestations
<1 IU/dL	Severe	Spontaneous joint and muscle bleeding; bleeding after injuries, accidents, and surgery
1-5 IU/dL	Moderate	Bleeding into joints and muscles after minor injuries; excessive bleeding after surgery and dental extractions
>5-40 IU/dL	Mild	Bleeding after surgery, dental extractions and accidents

Where normal is 100 IU/dL of FVIII/FIX (100%), as defined by the current World Health Organisation International Standard (White *et al.*, 2001).

1.2.3.1. Variability in clinical phenotype

The clinical phenotype in part is related to the baseline factor VIII or IX level, but variability in clinical phenotype is also seen among severe haemophilia patients. About 10-15% of patients with FVIII levels of < 1.0 IU/dL have clinically mild disease in terms of frequency of bleeds and extent of arthropathy. In addition, in some

patients from the same family, carrying the same gene defect, the bleeding tendency may not be identical. The mechanism of this phenotypic heterogeneity is not clearly understood (Dargaud *et al.*, 2004).

Recent studies have suggested that the presence of prothrombotic factors e.g. FV Leiden G1691A and prothrombin G20210A polymorphisms, can modify the clinical phenotype in haemophilia (van Dijk *et al.*, 2004). Increased levels of other procoagulant factors shown to confer an increased risk of thrombosis can also be potential modifiers of the haemophilia phenotype. Several studies have confirmed that higher levels of factors II, V (Mary, 2007), VIII (Koster *et al.*, 1995), IX (Vlieg *et al.*, 2000), and XI (Meijers *et al.*, 2000) are associated with venous thromboembolism (VTE) risk. Other reports suggest that FXII deficiency is related to risk of thrombosis (Gallimore *et al.*, 2004).

Platelets occupy a central role in the maintenance of haemostasis by adhering to sites of vascular injury and facilitating thrombin generation, leading to the formation of a fibrin clot. Individuals differ greatly with respect to platelet function, and platelets from different individuals differ inherently in their ability to support thrombin generation. There is evidence supporting the role of platelets and platelet-related factors in modifying bleeding tendency in patients with haemophilia (Dargaud, *et al.* 2006; Yee, 2006). Procoagulant microparticles are increasingly recognised as important mediators of haemostasis and thrombosis (Mackman, 2006). Platelet-derived microparticles demonstrate at least a fourfold variation in healthy individuals ($n = 15$) (Berckmans *et al.*, 2001) and even greater heterogeneity among haemophilia patients ($n = 79$) (Proulle *et al.*, 2005).

1.2.4. Diagnosis of haemophilia

Haemophilia is diagnosed either because of a known family history or after presentation with bleeding. The hallmark of severe haemophilia is spontaneous bleeding into joints and muscles that can be painful and destructive if inadequately treated. Most children with severe haemophilia experience their first bleed into a joint by the age of four, but many bleed from other sites before this age. Moderate haemophilia is diagnosed in most cases by the age of five, but mild haemophilia may be diagnosed much later in life after trauma or surgery. Therefore an accurate personal and family history is a crucial first step in the diagnosis of any bleeding disorder.

The activated partial thromboplastin time (APTT) has been used as a screening test for coagulation factor deficiencies since the early 1950s (Langdell *et al.*, 1953). The modern APTT test is a recalcification clotting time of citrated plasma with added surface contact activation (by either kaolin, celite, silica or ellagic acid) and a source of phospholipid. This is especially sensitive to coagulation abnormalities of the “intrinsic” pathway (factors VIII, IX, XI, and XII). Isolated prolongation of the APTT indicates a deficiency of one or more coagulation factors or inhibition of the coagulation process by heparin, lupus anticoagulant or specific factor inhibitors (Tripodi & Mannucci, 2006).

1.2.4.1. One-stage APTT factor assay

The basis of this bioassay is that of an APTT is performed on various dilutions of a standard and the sample, which are made in buffer and added to the specific factor deficient plasma (e.g. FVIII/FIX deficient plasma), to investigate the reduced or missing clotting factor. By plotting on a graph the time taken to clot for a known amount of the factor (e.g. FVIII/FIX), a standard curve is produced. The clotting time of at least three dilutions of the test plasma can be measured from the standard curve. An important prerequisite for this assay is parallelism between the standard curve and dilutions of patient plasma. Non-parallelism indicates presence of an inhibitor or very low level of the investigating factor. This assay is the most commonly used in the clinical laboratory due to the relatively low cost and automation of the assay (Goodnight & Hathaway, 2001).

1.2.4.2. Two-stage assay

The two-stage assay was developed by Biggs and colleagues in 1955. In the first stage, FVa and FXa are generated in an amount which is related to the sample's FVIII activity. In the second stage, prothrombin and fibrinogen are added, usually in the form of normal plasma, and the clotting time is recorded (Biggs *et al.*, 1955). This method makes no use of FVIII deficient plasma. It is claimed to show a better precision in general and also to be more sensitive than the one-stage method. However, it is more cumbersome to perform; hence it is not commonly used today (Barrowcliffe, 2004).

1.2.4.3. Chromogenic assay

The availability of a chromogenic substrate for FXa triggered the development of chromogenic methods for factor VIII activity and a specific method was published in 1985 (Rosen *et al.*, 1985). The assay principle is similar to the two-stage assay in being based upon an incubation step for generation of factor Xa and with no need of FVIII deficient plasma but rather utilizing purified bovine factors IXa and X. However, instead of using a fibrin clotting time as the end-point, the amount of factor Xa is determined from the hydrolysis of chromogenic substrate. The amount of generated factor Xa is directly proportional to the FVIII activity. This method has a greater precision and lower inter-laboratory variability than the one-stage assay with 2-7% coefficient of variation (CV) (van Dieijen *et al.*, 1987), as compared to one-stage assays which have CV of 7-10% (Raut *et al.*, 2001). The chromogenic assay is not widely used in the clinical setting due to the high cost of chromogenic substrates (Girolami & Vettore, 2009).

1.2.4.4. Discrepancies between methods

There are a number of reports on discrepancy between methods for measuring FVIII activity level, particularly in mild HA patients. In a comprehensive survey by Duncan *et al.* (1994), where the one-stage and the two-stage clotting methods were used in parallel, there were a number of index patients and also their affected family members who showed significantly higher results with the one-stage method than with the two-stage method, the latter method correlating much better with the clinical severity. Later, several single point mutations causing alterations of amino acids in the A1, A2 and A3 domains were identified in a number of those families, and those mutations

were not found in patients who showed similar results in the two methods (Pipe *et al.*, 2001; Rudzki *et al.*, 1996).

More recently, a discrepant result was also reported between the one-stage method and the chromogenic method, where the one-stage method showed a lower result and in this case correlated better with the clinical picture (Rodgers *et al.*, 2007). In the one-stage FVIII assay, where the physiological concentrations of thrombin activate the physiological concentrations of FVIII, the time between the first appearance of catalytic amounts of thrombin and a measurable clot may be as little as 15–20 s. Under these conditions, the assay will be extremely sensitive to altered thrombin binding or cleavage of FVIII. In contrast, in the chromogenic assay, an excess amount of thrombin activates diluted FVIII in a much longer time compared to the one-stage assay. Thus any altered affinity of thrombin towards FVIII will be overcome by this high thrombin concentration and activate FVIII (Oldenburg & Pavlova, 2010).

1.2.5. Treatment of haemophilia

Severe haemophilia patients may bleed spontaneously into joints and therefore require treatment, to replace the missing or dysfunctional factor. Bleeds can be treated as they occur (on demand) or treatment can be given regularly to prevent bleeds occurring (prophylaxis). By maintaining FVIII/FIX at a low level, spontaneous bleeding into the joints can be avoided. Treatment, when started at a young age, can prevent the development of arthropathy (van den Berg *et al.*, 2001). In patients with mild or moderate haemophilia treatment would be usually needed after injury or before a

surgical procedure. There are essentially two types of factor concentrates, plasma derived and recombinant.

A major challenge to the treatment of haemophilia is the development of inhibitors to the replacing factor. An inhibitor is a polyclonal inhibitory immunoglobulin G antibody directed against FVIII or FIX. It is estimated that the incidence of antibody development in persons with severe or moderate HA is between 20% and 33% (Lee *et al.*, 2005).

Among persons with haemophilia B, inhibitors are much less frequent, affecting only 1 to 6 %. However, the development of factor IX inhibitors can be associated with a mild or severe allergic reaction during factor IX administration. There is a high-risk period for the development of inhibitors during the first 50–100 days of exposure to infused factor. Historically, the majority of inhibitors have been reported to develop during childhood, at an average age of 10 years. A number of prognostic factors have been proposed such as genetic, environmental, and the type of factor concentrates (Hoffbrand *et al.* 2006).

1.3. Thrombin generation test

Thrombin generation is the central event in blood coagulation: Insufficient thrombin leads to an increased risk of bleeding, while unregulated thrombin generation predisposes to thrombosis. Whilst the PT and APTT are useful tests in identifying bleeding risk, they are of little value in predicting thrombotic risk, as they do not reflect overall thrombin generation. Figure 1.6 illustrates two haemostasis reaction

curves; a clot formation curve (e.g. PT or APTT), and a thrombin generation curve. For blood to clot, *in vivo*, it is only necessary for a small proportion of the available prothrombin to be converted to thrombin, with > 95% of thrombin formation occurring after this point (Baglin, 2005; Mann *et al.*, 2003b).

The thrombin generation test (TGT) has been used for more than 50 years since its development by Macfarlane and Biggs (1953) for use in whole blood and simultaneously by Pitney and Dacie (1953). Both methods were similar in that blood or plasma was placed in glass tubes with saline, subsamples were removed into a fibrinogen solution and the time taken for the fibrinogen to clot was measured.

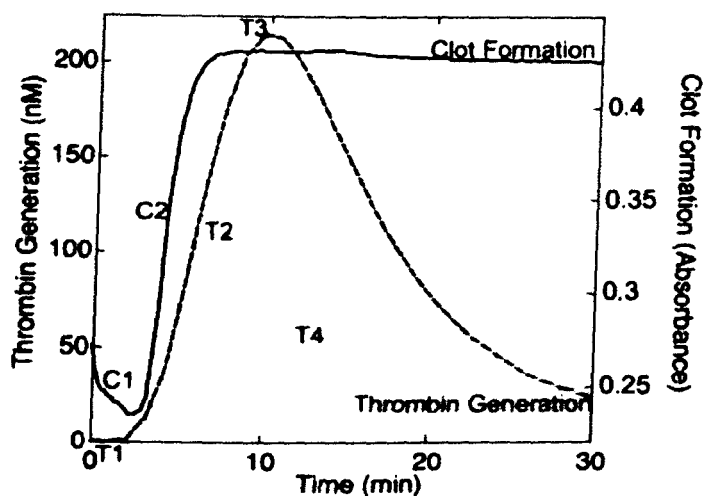


Figure 1.6 Comparison of clot formation and thrombin generation curves

TGT (dotted red line) can be described in phases: Lag (T1), maximum rate (T2), peak (T3) and total free thrombin produced (area under the curve, T4). Fibrin clot formation (blue) occurs concurrent with thrombin generation. In contrast to thrombin generation, the lag phase in the clot formation curve (C1) is a complex function reflecting initial thrombin generation, initial fibrin formation in an *in vitro* reaction tube. All clotting based coagulation assays detect this point as the end point of clot formation; (C2) occurs during thrombin's burst phase when the thrombin concentration is increasing, which is not detected in fibrin clot end point tests (Wolberg, 2007).

In the 1980s Hemker and colleagues developed this assay further by introducing some modification into the TGT (Hemker *et al.*, 1986). Initially Hemker *et al.* used the

chromogenic substrate and the plasma sample was defibrinated. However, the method was still a two-stage assay and therefore cumbersome. Sub sampling onto a chromogenic substrate was the first method in which thrombin was measured directly. Thrombin is neutralised predominantly by AT and α_2 -macroglobulin (α_2 -M). The technical problem was that the thrombin- α_2 -macroglobulin complex could cleave chromogenic substrates; therefore, the thrombin generation curve was the result of combined activities of free thrombin and thrombin- α_2 -macroglobulin complex on the chromogenic substrate (Figure 1.7). In order to calculate the area under the free thrombin curve (the physiological enzymatic work potential) a mathematical computer program was used to calculate the free thrombin activity from the thrombin generation curve (Hemker *et al.*, 1993). This was later designated the endogenous thrombin potential (ETP) (Hemker & Beguin, 1995).

The method still required defibrination of the plasma sample and it was realised that removal of fibrinogen has an adverse effect on the thrombin generation curve with a lower thrombin peak and a higher end-signal from the thrombin- α_2 -M complex (Hemker *et al.* 2000).

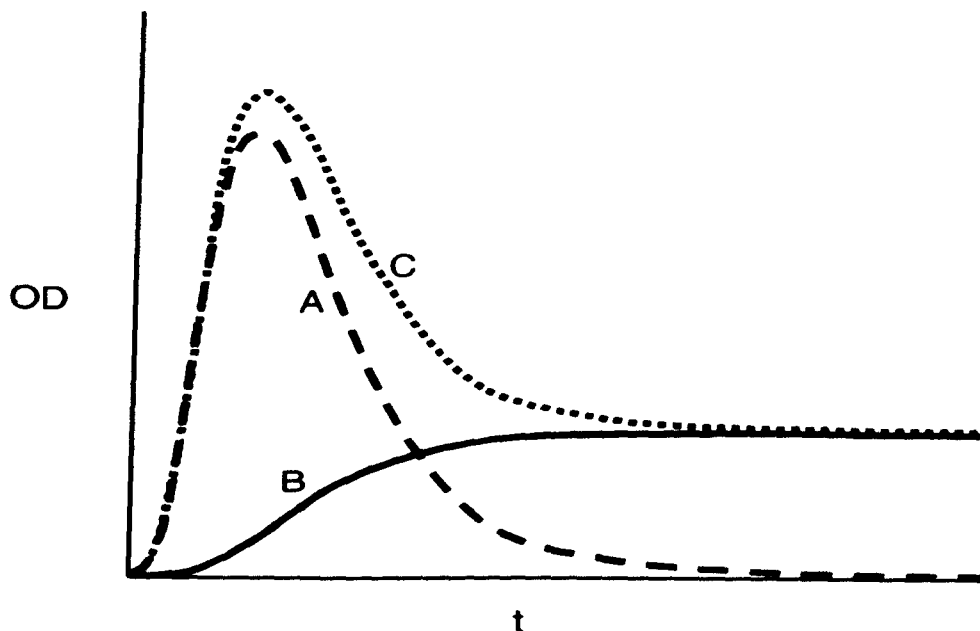


Figure 1.7 Thrombin generation curve with α_2 -macroglobulin

TGT curve measured by cleavage of a chromogenic substrate (curve C) is combined activity of the thrombin- α_2 -macroglobulin complex and free thrombin. Curve B is the thrombin- α_2 -macroglobulin complex activity only. Curve A is the area under the free-thrombin activity designated the Endogenous Thrombin Potential (ETP, nmol/min). OD, optical density for chromogenic substrate; t , time. (Baglin, 2005).

A further development of measurement of the thrombin generation was the use of a slow reacting substrate, which permitted continuous registration of thrombin activity in the primary reaction tube (Hemker *et al.*, 2000a). This converted the TGT method to a much simpler one-stage assay. The replacement of the chromogenic substrate with a slow reacting fluorogenic substrate enabled continuous measurement of thrombin generation without the need for defibrination. The calibrated automated thrombogram (CAT) was later introduced (Hemker *et al.*, 2003). In the CAT method the splitting of a fluorogenic substrate is monitored by comparing it to a constant known thrombin activity in a parallel, non-clotting sample.

Because of the use of fluorescence as a detection mechanism turbid media can be studied. Therefore the role of the platelets, fibrin, fibrin-platelet interaction and diffusion-controlled mechanisms are reflected as well. Thrombin generation can now

be performed on platelet poor plasma (PPP) as well as platelet rich plasma (PRP), as the signal from the fluorophore is not quenched by turbidity (Hemker *et al.*, 2000b).

1.4. Thromboelastometry

Thromboelastometry was first described by Hartert in 1948. In the original thromboelastogram a whole blood sample was placed into a cuvette, which rotated gently back and forth with a cycle time of 6/min. The viscoelastic changes that occur during coagulation were recorded (Luddington, 2005). Results obtained by classical thrombelastography (TEG) are dependent on the activity of the plasma coagulation system, platelet function, and fibrinolysis. Therefore, application of TEG could have several advantages compared with standard coagulation analysis that reflect only a part of the haemostatic process and additionally include partially unphysiologic activators.

With the advent of computer-assisted equipment, this technology is gaining more and more relevance in the clinical assessment of bleeding and thrombotic conditions (Rivard *et al.*, 2005). Recently, a modified rotational thromboelastometry analyzer (ROTEM[®], Pentapharm, Munich, Germany), has been marketed that overcomes some of the limitations of classical TEG. For example, by using an electronic pipette, reproducibility and performance has increased.

During ROTEM[®] measurements, the axis holding the pin, which serves as the sensor, is rotated alternately to the right and left at 4.75° by means of a spring mechanism. The rotations are optically monitored by means of a mirror, a light source, and a light

beam detector, and are eventually converted into a real time measurement by an integrated computer system (Figure 1.8). Upon measurement, initially, the rotation of the pin is unhindered, which is represented at this stage by a straight line in function of time. In ROTEM[®] this time it is defined as coagulation time (CT).

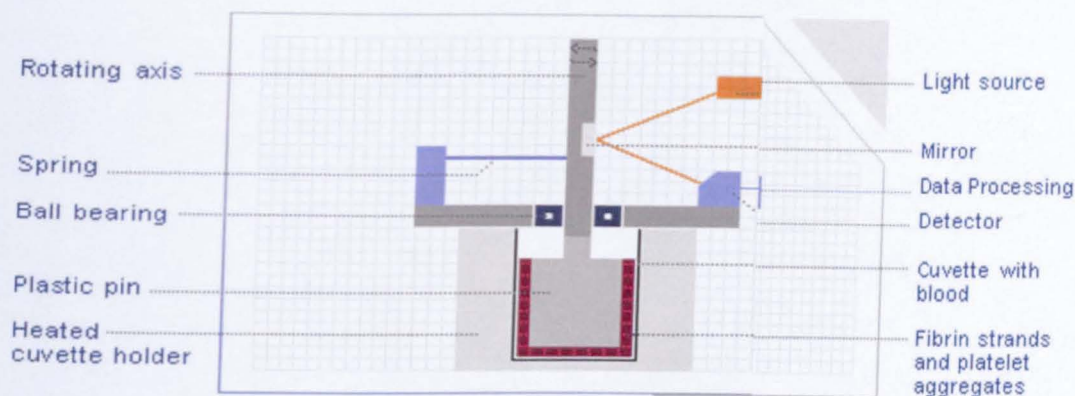


Figure 1.8 ROTEM[®]

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With the initiation (by contact activation) of the coagulation process, the rotational movements are influenced by adhesion of fibrin threads developing between the cup and pin. The period of time to reach a width of 20 mm is the clot formation time (CFT). CT and CFT are usually expressed in seconds. The maximum width of the thromboelastogram corresponds to the maximum clot firmness (MCF). The maximum width of the thromboelastogram is measured in millimetres (Figure. 1.9a)

On reaching the MCF, the secondary coagulation phase is completed. Subsequently, the amplitude is reduced by fibrinolytic processes.

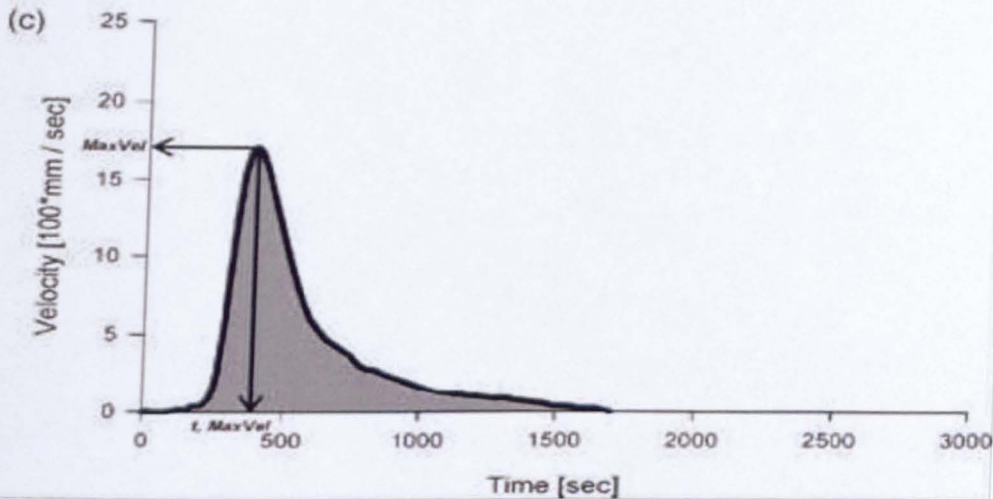
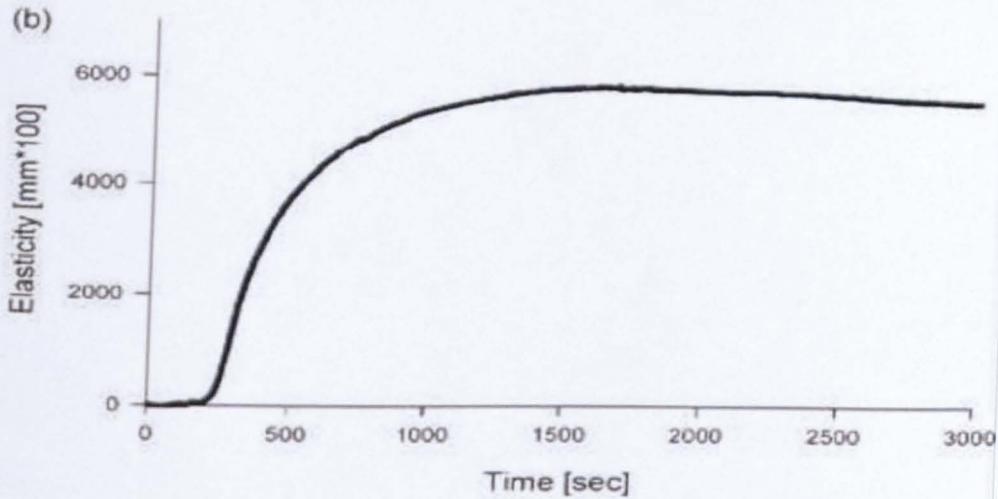
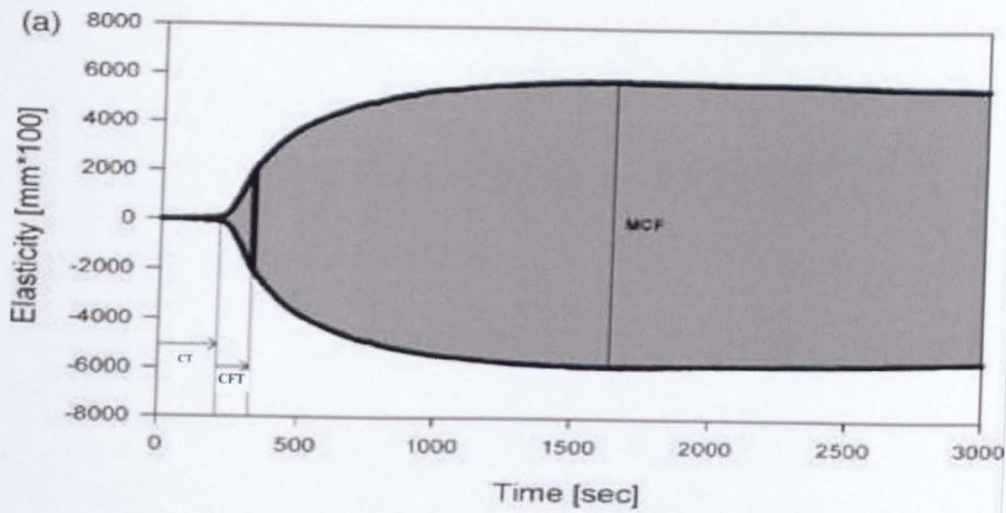


Figure 1.9 ROTEM® tracing

Principal characteristics of thromboelastometry software parameters and first derivative parameters of whole blood clot formation. (a) Standard thromboelastometry tracking. CT is the clotting time, CFT is the clot formation time, and the MCF is the maximum amplitude of clot formation. (b) ROTEM® tracking imported into the ROTEM® software (CalcuRo). (c) Velocity profile, the first derivative of the thromboelastometry course. Maximum velocity (MaxVel, marked with a horizontal arrow) is the maximum rate of whole blood clot formation. Time to maximum velocity (tMaxVel, marked with a perpendicular arrow) is the time until maximum velocity occurs. The area under the velocity curve (AUC, marked with gray colour) indicates the maximum clot formation, an indirect measurement of clot strength. Taken from (Sørensen *et al.*, 2003) with permission.

In addition, α angle (α°) may be used, which demonstrates the kinetics of clot formation. The α angle represents the speed at which a solid clot forms and is primarily influenced by platelet function, but coagulation factors contribute to a certain extent, especially fibrinogen. Apart from the parameters CT, CFT, α° , and MCF, there are several further parameters such as the maximum lysis (ML) and the fibrinolysis index (LI), which may be measured after 30, 45 and 60 min, respectively, describing the fibrinolytic process (Sørensen *et al.*, 2003).

Thromboelastometry curves can be analysed using a software program provided with the ROTEM[®] analyzer, which was first described by Sørensen *et al.* (2003). The raw data is imported into ROTEM[®] software CalcuRo (Pentapharm GmbH, Munchen, Germany) for analysis of maximum velocity (MaxVel), time to maximum velocity (tMaxVel), and area under the curve (AUC) (Landskroner *et al.*, 2005) (Figure 1.9.c).

1.5. Clot waveform

The MDA[®] analyser (Organon Teknika, Cambridge, UK) is a fully automated coagulometer, which performs coagulation assays by using a variable wavelength photo-optical detection system. During the performance of routine clotting assays such as APTT and PT, it is possible to obtain a continuous measurement of the changes in light transmittance that occur as the fibrinogen in the test citrated plasma sample undergoes polymerisation to form a clot. The resultant photo-optical data profiles obtained by continuous monitoring are called waveforms caused by their sigmoid patterns, which can be used to define specific events that occur prior to, during and following initiation of the clotting reaction (Downey *et al.*, 1997).

The waveform is mathematically processed by a software algorithm to derive a set of 10 parameters (Table 1.2) such as coagulation velocity (Min1) by measuring the percentage difference in light transmittance divided by time difference in second (%T/s), and acceleration (Min2), calculated based on difference in percentage of change in light transmittance over time in squared second (%T/s²) (Figure 1.10) (Shima *et al.*, 2002).

Using this approach, the waveform of an APTT assay can be divided into three segments or phases: pro-coagulant phase, coagulation phase, and post-coagulation phase. These phases are characterised by a set of parameters that define:

- The timing of individual events during the reaction.
- The rate at which these events occur.
- The magnitude of the change.

Table 1.2 Clot waveform parameters

Phase	Parameter	Description
Pro-coagulation (a-b)	Slope 1 Delta 1	Initial slope of line fit to data from a (point of addition of activating reagent) to b (initiation of coagulation) Amplitude of signal change from point a to b
Coagulation (b-d)	Index min2 Min2 Index min1 Min1 Index max2 Max2	Time at point b (onset of coagulation) Minimum value of second derivative (acceleration at point b) Time at point c (coagulation midpoint) Minimum value of first derivative (coagulation velocity) Time at point d (end of coagulation phase) Maximum value of the second derivative (deceleration point)
Post-coagulation (d-e)	Slope3 Delta	Slope of line fit from d to e (end of acquisition time) Amplitude of total signal change between point a and e

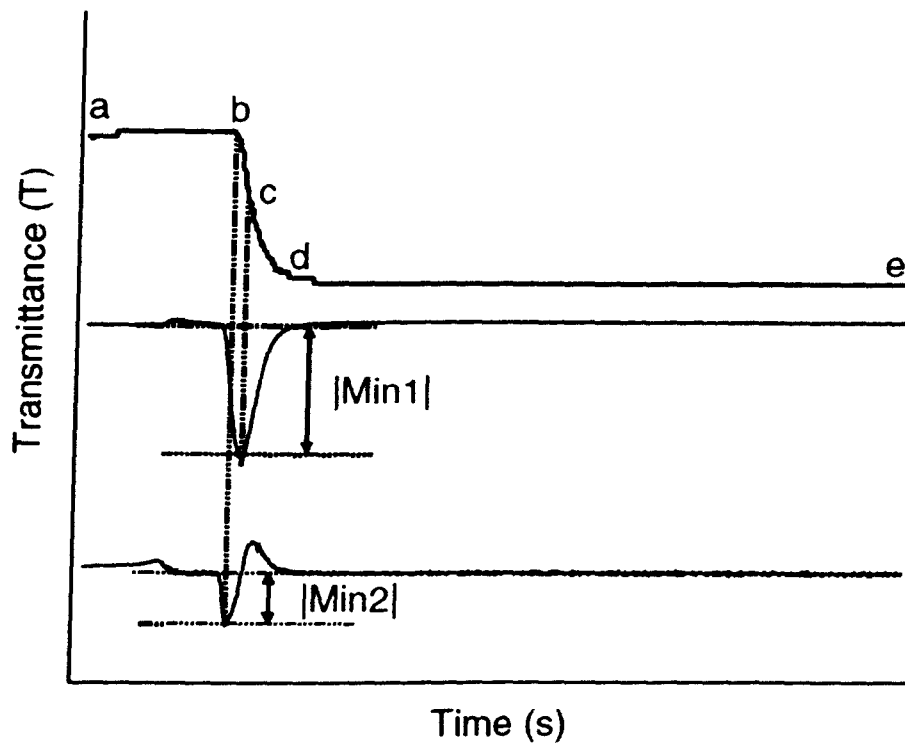


Figure 1.10 Clot waveform.

In the normal waveform, the upper trace shows the changes in transmittance (T) observed over time (t) during the performance of an APTT. The middle trace shows the first derivative (dT/dt) from the transmittance data and is denoted Min1. The lower trace shows the second derivative (d^2T/dt^2) and is denoted Min2. Point a is the start of transmittance by addition of activating reagent. Point b indicates the initiation of coagulation. Point c is the coagulation midpoint. Point d is the end of coagulation phase. Point e is the end of acquisition time. The absolute value of Min1 shows the maximum coagulation velocity and the absolute value of Min2 shows the maximum coagulation acceleration (adapted from Shima (2008)).

The data provided by clot waveform analysis reflect fibrinogen to fibrin conversion. Fibrinogen is the ultimate substrate for thrombin in the coagulation cascade, and it is likely, therefore that clot waveforms also reflect thrombin generation (Shima *et al.*, 2008). Thus clot waveform analysis may be useful for the investigation of the clinical phenotype of individual patients.

A comparison of clot waveform analysis and TGT in haemophilia A and B demonstrated a significant correlation between the clot waveform and TGT parameters (Shima *et al.*, 2008).

1.6. Aims

1.6.1. Rationale for current study

Traditional coagulation assays, such as APTT, are usually performed in platelet poor plasma (PPP). They provide information only on the initial stage of clot formation. The end point of APTT-based assays is the formation of a fibrin clot by the initial generation of thrombin. However, more than 95% of thrombin, which is produced during the propagation phase of coagulation, remains undetected in these assays (Bates & Weitz, 2005). In patients with documented haemorrhagic disorders, only modest alterations of thrombin production occur during the initiation phase, while depression of thrombin production during the propagation phase is the hallmark of these congenital bleeding diseases as assessed by the laborious sub-sampling chromogenic method of thrombin generation (Hemker & Beguin, 1995).

The sensitivity of APTT based coagulation factor assays is limited to 1.0 IU/dL activity of the relevant coagulation factor level (Scharer & Schramm, 2007); therefore, the phenotype of patients with a factor level of < 1.0 IU/dL and mild bleeding frequency remains inconclusive by the current assays. Further clot waveform analysis is sensitive to changes of factor VIII concentration < 1.0 IU/dL (Shima *et al.*, 2002). It is conceivable that global assays incorporating platelets and other cellular components might amplify the effects of factor VIII < 1.0 IU/ dL on thrombin generation.

There has also been growing interest as to whether global tests, including the TGT, thromboelastometry, clot waveform analysis, that assess the interaction of the procoagulants and anticoagulants on generation of thrombin, and the ability of

platelets to support this thrombin generation may better define the bleeding tendency in patients with haemophilia. This would have an impact on the immediate management of patients, and potentially contribute to individualising treatment based on the patient's coagulation phenotype.

It has been reported that thrombin generation in mild and moderate haemophilia is less impaired than in patients with severe haemophilia (Beltran-Miranda *et al.*, 2005; Dargaud *et al.*, 2005). Accordingly, differences in thrombin generation between patients with severe haemophilia may explain the observed differences in clinical presentation.

Preliminary data, (Chantarangkul *et al.*, 2003; Dargaud *et al.*, 2005; Dargaud *et al.*, 2007; Lewis *et al.*, 2007; Luddington & Baglin, 2004) show that the analysis of parameters in thrombin generation is a useful tool to detect bleeding tendency in haemophiliacs. However, these studies have been performed on PPP; therefore the effect of platelets on thrombin generation has not been taken into account. Furthermore, none of the studies published when the project was conceived correlated global assays to the type of mutation or the clinical phenotype.

1.6.2. Aims

The aim of this study was to critically evaluate the role of global assays in the investigation of haemophilia disorder, including the influence of preanalytical variables.

1.6.3. Objectives

The global assays chosen for this study, included modified rotating thromboelastometry (ROTEM[®]) as described by Sørensen *et al.* (2003), performed on whole blood, with the whole blood being activated either by the contact pathway or through the use of tissue factor.

In house thrombin generation in platelet poor and platelet rich plasma was also performed with appropriate concentration of tissue factor, to assess the heterogeneity of thrombin generation. A commercial thrombin generation assay as described by Hemker *et al.* (2003), the calibrated automated thrombogram (CAT) was also used to compare with the in-house method.

Clot waveform analysis, based on light transmission on the MDA analyser, was also incorporated to provide a sophisticated analysis of the fibrin clot formation beyond clot detection as a surrogate marker of thrombin generation (Shima *et al.*, 2002).

The prevalence of thrombophilic defects, such as FV Leiden and prothrombin gene mutation (G20210A) that can potentially influence global coagulation assays was also evaluated. The global assays were compared with each other, and to conventional factor assays to identify the most sensitive parameters of disease severity.

Furthermore, these sensitive parameters of global assays were correlated to the type of mutation and the clinical phenotype as described by the clinicians in the Haemophilia Centre, to evaluate their utility in predicting the bleeding tendency.

1.6.4. Hypothesis

Global assays including TGT, ROTEM[®], and clot waveform analysis may reflect the clinical phenotype of individuals with haemophilia A and B better than current conventional methods such as clotting factor assays.

The existence of other defects in haemostasis system may modulate the coagulation potential of individuals toward hypo/hypercoagulability. In haemophilia this may influence the bleeding phenotype of patients.

Chapter 2 **Materials and Methods**

2.1. Patient Material

Patients studied were registered at the Katharine Dormandy Haemophilia Centre and Thrombosis Unit, Royal Free Hospital, London, UK. All patients had been previously identified as having haemophilia A or B. All patient material was collected with informed consent and the study conducted in accordance with the International Conference on Harmonisation (ICH) and World Health Organisation (WHO) Good Clinical Practice standards, after local ethical committee (the Royal Free hospital ethics committee; reference number: 04/Q050/120) approval.

2.1.1. Inclusion criteria

- Patients with Haemophilia A or B
- Willingness to participate in the study by informed written consent
- Age ≥ 16 years old
- Most recent concentrate treatment 72 h before sample collection for haemophilia A patients and 5 days for haemophilia B patients (the median half-life for FVIII and FIX are 12 (van Dijk *et al.*, 2005b) and 19 h respectively (Poon *et al.*, 1995); the washout period is considered 3 to 5 half lives (Lee *et al.*, 2004).

2.1.2. Exclusion criteria

- Patients with an inhibitor towards Factor VIII or IX
- Severe HA or HB patients with > 1 IU/dL FVIII/FIX activity level

(which could be the treatment factor concentrate)

- Patients with liver disease (liver function tests included alanine transaminase (ALT), aspartate transaminase (AST) and γ -glutamyl transpeptidase (γ GT), which were performed at the time of enrolment by taking an extra blood sample and analysed in the Biochemistry laboratory of RFH)
- Patients with high lipid level (elevation of lipids and/or cholesterol levels may increase the total amount of thrombin generation (Aoki *et al.*, 1997; Takayuki *et al.*, 2001; Vincenzo *et al.*, 1997); lipid profile was analysed at the same time with liver function test)
- Inability or unwillingness to comply with protocol (including patients that have difficulty understanding written or verbal English).

There were 97 patients who were enrolled into the study and who were previously diagnosed with severe HA ($n = 49$), moderate HA ($n = 11$), mild HA ($n = 16$), severe HB ($n = 14$), moderate HB ($n = 3$), and mild HB ($n = 4$). The diagnosis in these patients was based on the clinical presentation, the baseline factor level (Appendix 1), which was performed by one-stage factor assay, and relevant factor mutational analysis. The individuals were diagnosed at the Katharine Dormandy Haemophilia Centre and Thrombosis unit (KDHCTU) of the Royal Free Hospital, London, UK. In order to obtain the baseline factor level of individuals, patients were asked to omit treatment for 72 h for Haemophilia A, and 5 days for Haemophilia B.

A number of individuals were excluded due to slow clearance of treatment material (13 severe HA, seven severe HB) or abnormal liver function tests. Also as result of a

technical problem with the ROTEM[®] analyser, six severe HA and one severe HB patients were not included in the ROTEM[®] study. A brief description of study subjects has been given in each chapter.

2.2. Control Subjects

A group of healthy subjects from the Royal Free Hospital staff were used as controls and bled after informed consent to allow calculation of reference ranges for TGT, ROTEM[®], and clot waveform analysis. All samples were collected with informed consent.

2.3. Sample Collection

Collection tubes, Monovette[®], were purchased from Sarstedt, Leicester, UK. Whole blood samples were collected by venepuncture into Monovette[®] tubes containing 0.106 M (3.8%) trisodium citrate in a ratio of 1 part anticoagulant to 9 parts blood for analysis. Monovette[®] tubes were spiked with 20 µg/mL corn trypsin inhibitor (CTI) (see section 2.4.8). Whole blood was also collected into Monovette[®] tubes containing ethylene diamine tetra acetic acid (EDTA) for full blood count (FBC), and Monovette[®] gel tubes to investigate liver function and lipid profile of each patient. FBC was performed by the Haematology Laboratory, and liver function tests and lipid profiles were analysed by the Clinical Biochemistry Laboratory, at the Royal Free Hospital.

2.3.1. Sample processing and storage

Citrated whole blood (CWB) for investigation by ROTEM[®] was rested for 30 min (as required by manufacturer for testing on citrated whole blood) before performing the test, according to manufacturer's instructions. CWB samples were centrifuged at 150 g for 15 min to obtain platelet rich plasma (PRP). A platelet count was performed in the Haematology Laboratory, using an ADVIA[®]120 (Bayer, Newbury, UK), and adjusted by autologous platelet poor plasma (PPP) to obtain a platelet count of $150 \times 10^9/\text{L}$. The remainder of citrate samples collected by Monovette[®] were double spun at 2000 g for 15 min to obtain PPP, which were then aliquoted into microtubes and stored at -70°C for remainder of the coagulation investigations.

For TGT, PPP was centrifuged at 6000 g for a further 2 min to obtain ultra spun PPP, free from platelets and micro-particles. The preliminary experimental result of TGT showed that double spinning of samples would not remove platelets or platelet debris. This was proven when the double spun plasma samples were indirectly tested for the presence of phospholipids by kaolin clotting time (KCT) test. The KCT is essentially an APTT test without added phospholipids. The double spun plasma samples had significantly shorter KCT than the triple spun plasma samples, therefore, the third spin, which was at 6000 g for 2 min, was added to the study protocol.

2.4. Coagulation Investigations

Prothrombin time (PT-Fibrinogen HS Plus, Haemostasis Instrumentation Laboratory (IL[™]) Ltd, Warrington, UK), APTT (SynthASil, IL[™]), thrombin time (TT) (Thrombin Time, IL[™]), and Clauss fibrinogen (Fibrinogen-C, IL[™]), assays were performed on an

ACL™ TOP (IL™) analyser as routine for each patient on the day of testing. The remainder of the investigations were done after being batched.

2.4.1. Intrinsic and extrinsic factor assays

One stage APTT and PT based factor assays were analysed, using an ACL™ 300R (IL™). All factor assays were performed in an identical fashion using appropriate factor deficient plasma. One stage factor assays were performed for the intrinsic factors VIII, IX, XI, XII; and extrinsic factors and common pathway II, V, VII, and X on the ACL™ 300R. Patient's PPP was diluted 1:10, 1:20, and 1:40 on Owren's Buffered Saline (OBS) and compared to a three-point standard curve.

For intrinsic factor assays, 100 µL of APTT reagent (APTT lyophilised reagent IL™), 100 µL of factor deficient plasma (Technoclone, Dorking, UK) and 100 µL of diluted test or standard (CRYOcheck™ pooled normal plasma (Alpha Laboratories Ltd, Eastleigh, UK) were warmed at 37°C for 5 min, then 100 µL 0.25 M CaCl₂ (IL™) was added and the time taken for the mixture to clot was recorded. For the extrinsic factor assays, 100 µL of diluted test or standard was warmed at 37°C for 2 min then 200 µL of PT reagent was added. The time taken for the mixture to clot was measured in seconds by ACL™ 300R analyser. All sample results (three point dilution) were plotted on graph paper (log-linear graph paper for factors VIII, IX, and XI, and log-log graph paper for factors II, V, VII, X, and XII) against a 3 point standard curve. The CRYOcheck™ is a normal reference plasma collected from a minimum of 20 carefully screened donors and calibrated against international reference standards, and values for haemostatic parameters are assigned. At the KDHCTU laboratory,

CRYOcheck™ values for haemostatic parameters is further evaluated, and calibrated against British or international standards for the use on ACL™ analysers.

2.4.2. Chromogenic FVIII Assay

FVIII was measured using Coatest C/4 kit Chromogenix (Quadrach, Epsom, Surrey). The kit provided lyophilised FX/FIXa reagent, and chromogenic substrate (S-2765 Benzoyl-Ile-Glu(γ-OR)-Gly-Arg-pN), for reconstitution with distilled water. Liquid phospholipid (PL) reagent is also provided. The dilution buffer was made by diluting stock buffer 1 in 10 with distilled water.

Test, quality control and standard sample dilutions were made in replicate with buffer to 1 in 50, 1 in 100, and 1 in 200. Samples were then loaded onto an ACL™ 300R analyser and kit reagents added. The ACL™ 300R was set up in the following manner: 25 µL of sample was incubated with 75 µL of combined reagent for 300 s, subsequently 50 µL of chromogenic substrate was added, and optical density (OD) at 405 nm was measured following a further 220 s incubation period.

2.4.3. Physiological inhibitors of coagulation

2.4.3.1. Antithrombin (AT) Activity Assay

The assay was performed on the ACL™ 300R (IL™) by an in-house chromogenic assay (Harper *et al.*, 1991).

Method:

Lyophilised bovine thrombin (Diagnostic Reagent Ltd, Thame, UK) reconstituted with one mL distilled water, then 40 μ L of reconstituted Bovine thrombin (1000 IU/mL), and 60 μ L sodium heparin (1000 IU/mL) were added to 10 mL working buffer to make a working enzyme reagent. Chromogenic substrate, S2238 (H-D-CHT-Abut-Arg-pNA), was reconstituted with 25 mL distilled water. The standard curve was prepared using four dilutions of 1:50, 1:75, 1:100, and 1:200 of the reference plasma (CRYOcheck™) in working buffer. Quality controls and test plasma were diluted 1:100 and 1:200 in working buffer. Using AT chromogenic programme on the ACL™ 300R the test was performed. AT, in the presence of heparin and excess amount of thrombin formed an antithrombin-thrombin-heparin complex. The residual thrombin catalysed the release of para-nitroaniline (pNA) from the chromogenic substrate. The rate at which pNA is released is measured photometrically at 405 nm and the AT levels in the plasma were inversely proportional to the change in optical density.

2.4.3.2. Protein C Activity Assay

Protein C (Hepner *et al.*) was measured using HemosIL™ PC activity kit (IL™). The IL™ PC activity kit uses a protein fraction derived from the venom of the southern copperhead snake *Agkistrodon contortrix contortrix*, which activates PC. The activated PC, in turn, hydrolyses a synthetic chromogenic substrate (S-2366 pyroGlu-Pro-Arg-pNA). The release of pNA is proportional to the activity of PC.

Method:

The kit consisted of a PC activator (lyophilised fraction of aforementioned snake venom), a chromogenic substrate (lyophilised S-2399), and a diluent (concentrated solution containing 0.9% sodium chloride).

The lyophilised PC activator and chromogenic substrate were each reconstituted with 2.5 mL distilled water and kept at room temperature for 30 min according to the manufacturer's instruction. The diluent was diluted 1 in 10 with distilled water. Using the ACL™ TOP PC activity test protocol, 56 µL of quality control/test plasma was mixed with 56 µL of PC activator and incubated for 4 min, before the addition of 56 µL of substrate reagent. The pNA released was monitored kinetically at OD of 405 nm.

2.4.3.3. Free Protein S Assay

Free PS was measured using a commercial latex free protein kits (Free PS, IL™), on an ACL™ TOP (IL™) according to the manufacturer's instructions, by measuring an increase in turbidity produced by the agglutination of two latex reagents. Purified C₄BP is adsorbed on the C₄BP latex in the presence of Ca²⁺. Free PS, in the test plasma, will move from the solution to the latex surface, and the amount of PS covering the latex will be proportional to the free PS concentration in the sample. The free PS-C₄BP latex triggers the agglutination reaction with the second latex reagent which is sensitised with a monoclonal antibody directed against human PS. The degree of agglutination will be directly proportional to the free PS concentration in the test sample.

Method:

The kit consisted of two reagents and one buffer: reagent R1 (latex polystyrene beads coated with purified human C₄BP), reagent R2 (latex polystyrene beads coated with a monoclonal antibody against human PS), and C₄BP buffer (Borax buffer containing bovine serum albumin).

The content of R1 reagent was dissolved with the whole content of C₄BP buffer (4 mL) and kept at room temperature for 30 min before being placed on the ACL™ TOP analyser. Using the ACL™ TOP PS activity test protocol, 12 µL of quality control/test plasma was incubated with 150 µL of reagent R1 for 130 s. Changes of OD at 405 nm were recorded for 300 s after adding 80 µL of reagent R2.

2.4.4. von Willebrand Factor Antigen (VWF:Ag)

The Enzyme Linked Immunosorbent Assay (ELISA) principle was adopted for two assays.

The following buffers were used for both ELISA methods:

Bicarbonate Buffer (0.05M)

1.59 g Na₂CO₃, 2.93 g NaHCO₃ in 1.0 L distilled water, pH 9.6

High Salt Wash Buffer with Tween (HSBT) (0.155M)

1.95 g NaH₂PO₄·2H₂O, 141.1 g NaCl, 13.4 g Na₂HPO₄·12H₂O, 10.0 mL Tween 20 in 5.0 L of distilled water, pH 7.4

Tag and Sample Dilution Buffer

15.0 g polyethylene glycol (PEG) 8000 in 500 mL HSBT

Substrate buffer (0.1M)

7.3 g citric acid, 23.87 g Na₂HPO₄·12H₂O in 1.0 L of distilled water, pH 5.0

NUNC maxisorb microtitre plates (Life Technologies Ltd, Paisley, Scotland) were used for VWF:Ag ELISA and Covalik microtitre plates (Life Technologies Ltd, Paisley, Scotland) were used for VWF collagen binding assay (VWF:CB). The optical density readings were determined by a Titertek plate reader (ICN, Flow Biomedicals LTD, Bucks).

Method:

Polyclonal anti VWF:Ag (Dako Ltd, Bucks, UK) was diluted 1 in 1000 in bicarbonate buffer. 100 μ L of diluted coat antibody was added to each well of microtitre plate. The plate was sealed and left at 4°C overnight. A seven point standard curve (125.00 to 6.25 IU/dL) was prepared using CRYOcheck™ pooled normal plasma, initially diluted 1:80 (125 IU/dL). Test plasma and quality control samples (CRYOcheck™ was used as a normal quality control, and control plasma P (Sysmex UK Ltd, Milton Keynes, UK), was used as an abnormal quality control) were diluted 1:100 and 1:200.

The coated plate was washed 5 times with HSBT. Test, quality control or standard dilutions were added in 100 μ L volumes, in duplicate, to the plate and incubated for one hour at room temperature. At the end of incubation the plate was washed a further five times with HSBT, after which, 100 μ L of diluted (1:8000) (in sample dilution buffer) horseradish peroxidase conjugated anti-VWF antibody (Dako) was added to each well of the plate. The plate was again incubated for one hour on a plate shaker at room temperature (18-25°C). Just before the end of incubation the substrate solution was prepared by dissolving one 10.0 mg orthophenyline-diamine (OPD) tablet in 15.0 mL substrate buffer. The plate was then washed for a final five times. Immediately before the next step, 7.0 μ L of 30% hydrogen peroxide was added to the substrate solution, 100 μ L of substrate solution was then added to each well at timed intervals

(one second). After 10 min the reaction was stopped by the addition of 100 μ L of 1.5 M sulphuric acid to each well at the same time interval. The absorbance of each well of the plate was read within 30 min at 492 nm. The plate reader software calibrated a VWF:Ag standard curve (optical density against IU/dL on a semi-log scale) and then calculated the mean of duplicate tests and the quality control results from the curve.

2.4.5. Collagen Binding Assay (VWF:CB)

Human placental type III collagen was diluted 1 in 200 in bicarbonate buffer. 100 μ L of the dilution was added to each well of the microtitre plate. The plate was sealed and left at room temperature overnight. A seven point standard curve (125.00 to 6.25 IU/dL) was prepared using CRYOcheck™ pooled normal plasma. Test plasma, normal quality control (CRYOcheck™) and abnormal quality control (control plasma P) samples were diluted 1:200 and 1:400.

The coated plate was washed 5 times with HSBT. Test, quality control or standard dilutions were added in 100 μ L volumes in duplicate to the plate and incubated for 1 h at room temperature. At the end of incubation the plate was washed a further five times with HSBT, after which, 100 μ L of diluted (1:4000) (in sample buffer) horseradish peroxidase conjugated anti-VWF antibody (Dako) was added to each well of the plate. The plate was again incubated for 1 h on a plate shaker at room temperature (18-25°C). Just before the end of incubation the substrate solution was prepared by dissolving one 10.0 mg orthophenyline-diamine (OPD) tablet in 15.0 mL substrate buffer. The plate was then washed for a final five times. Immediately before the next step, 7.0 μ L of 30% hydrogen peroxide was added to the substrate solution, 100 μ L of substrate solution was then added to each well at timed intervals (one

second). After 10 min the reaction was stopped by the addition of 100 μ L 1.5 M sulphuric acid to each well at the same time interval. The absorbance of each well of the plate was read within 30 min at 492 nm. The plate reader software calibrated a VWF:CB standard curve (optical density against IU/dL on a semi-log scale) and then calculated the mean test and the quality control results from the curve.

2.4.6. Factor VIII Antigen (FVIII:Ag) Assay

The FVIII: Ag was measured by an ELISA method, using a commercial assay Asserachrom[®] VIII:Ag kit (Diagnostica STAGO UK Ltd, Reading, UK).

Kit reagents:

Reagent 1: 16-well strip coated with mouse monoclonal anti-human FVIII F(ab)₂ fragment to capture FVIII.

Reagent 2: Mouse monoclonal anti-human FVIII antibody coupled with peroxidase to bind to the remaining free antigenic determinants of the bound FVIII.

Reagent 3: Tetramethylbenzidine < 1% substrate solution. The bound enzyme peroxidase is revealed by its action on the tetramethylbenzidine substrate.

Reagent 4: phosphate buffer as dilution buffer for calibrator, quality controls, and plasma samples.

Reagent 5: 20-fold concentration washing solution.

Reagent 6: freeze-dried human plasma containing a known amount of FVIII, to be used as a calibrator.

Reagent 7: freeze-dried human plasma containing a known amount of FVIII, to be used as a quality control.

Method:

The assay was calibrated with Reagent 6 diluted 1:10 with Reagent 4 (dilution buffer). Serial 1:2 dilutions were made from the starting solution by Reagent 4. Plasma samples and quality controls were diluted at two dilutions, of 1:10 and 1:20, with Reagent 4.

200 μ L of diluted calibrator (diluted in Reagent 4), quality controls, and test plasma were added in duplicate to the strip wells and incubated at room temperature for 2 h. All wells were washed with 1:20 diluted Reagent 5 (diluted with distilled water). After completely emptying the plate, 200 μ L Reagent 3 for exactly 5 min, then 50 μ L 1.0 M sulphuric acid were added to each well. After 15 min, the absorbance of each well was read at 450 nm. Log-log graph paper was used to draw the calibration curve (the calibrator values (%) on the X-axis and their corresponding absorbance values on the Y-axis). The absorbance values of the quality control and test plasma dilutions were interpolated and the value of FVIII:Ag level of each sample were derived from the calibration curve. Results from 1:10 dilutions were read directly from the curve; and those for 1:20 dilutions were multiplied by 2.

2.4.7. Factor V Leiden and Prothrombin gene mutation analysis

For both assay methods the DNA was extracted by the QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany) using citrated whole blood. Using a standard polymerase chain reaction (PCR) method the factor V_{Leiden} mutation (G1691A) was detected by PCR amplification of genomic DNA followed by Mnl I restriction enzyme digestion, as described previously (Bertina *et al.*, 1994).

The prothrombin mutation (G20210A) was determined by PCR amplification of genomic DNA followed by Hind III restriction enzyme digestion, as described previously (Poort *et al.*, 1996).

2.4.8. Thrombin Generation Test

Reagents:

HEPES Working Buffer (HWB): 0.476 g of 1.0 M 4-(2-hydroxyethyl)-1 piperazineethanesulfonic (HEPES) (Autogen Bioclear™, Calne, Wiltshire, UK), 0.812 g 1.0 M NaCl, and 0.5 g Bovine Serum Albumin (BSA) (Sigma, Poole, UK) dissolved in 100 mL distilled water to make 20 mmol/L HEPES, 140 mmol/L NaCl, 5.0 ml/L, pH 7.35.

HEPES Substrate Buffer (HSB): 0.476 g of 1.0 M HEPES, and 6.0 g of BSA were dissolved in 100 mL distilled water to make 20 mmol/L HEPES, 60 g/L BSA, pH 7.35.

Corn Trypsin Inhibitor:

CTI (Cambridge Bioscience Ltd, Cambridge, UK) is a specific inhibitor of human FXIIa. In order to eliminate activation of the coagulation cascade through contact activation it is necessary to inactivate/inhibit FXIIa prior to initiation of coagulation with a low concentration of TF. Contact pathway activation readily occurs in vitro when blood is exposed to artificial surfaces (i.e. blood collection tubes). The activation of contact pathway may also occur at the time of venepuncture, sample processing and centrifugation. Even when all precautionary measures to be taken to

illuminate pre-analytical variables, several studies have shown the necessity of inhibition of contact pathway at low TF concentration (Dargaud *et al.*, 2006b; Luddington & Baglin, 2004; van Veen *et al.*, 2009).

This study was conducted before the use of CTI in TGT was recommended as the standard of testing for patients with inherited bleeding disorders (Berntorp & Salvagno, 2008), and therefore samples were analysed with and without CTI to show the effect of CTI in TGT and ROTEM[®] studies. CTI was added to the sodium citrate Monovette[®] tubes just before collecting blood samples. The final concentration of CTI in the Monovette[®] was 20 µg/mL.

Phospholipid Vesicles Preparation

The PLs used were a mixture of 60% phosphatidyl choline (PL:PC), 20% phosphatidyl serine (PL:PS) and 20% phosphatidyl ethanolamine (PL:PE). The stocks reagents were purchased from Avanti Polar Lipids (Delfzyl, The Netherland).

For preparation of 1 mL mixture of 1 mM PL:PS-PL:PE-PL:PC (20-20-60):

Stocks: PL:PS (10.0 mg/mL; 2.5 mL; MW: 810.03; CHCl₃) = 12.34 mM

PL:PE (25.0 mg/mL; 4.0 mL; MW: 744.04; CHCl₃) = 33.60 mM

PL:PC (25.0 mg/mL; 4.0 mL; MW: 786.15; CHCl₃) = 31.80 mM

16.0 µL PL:PS + 6.0 µL PL:PE + 19.0 µL PL:PC was pipetted into a glass tube. Nitrogen-gas (BOC, Stratford, UK) was blown in the tube until all chloroform (CHCl₃) disappeared, after which, 1.0 mL buffer (140 mM NaCl, 20 mM HEPES, pH 7.35) was added. The resulting solution was a turbid suspension and had to be extruded by a mini-extruder (Avanti Polar Lipids, Delfzyl, The Netherlands), through

a 100 nm and 300 nm filter until the solution was clear and to make unilamellar phospholipid vesicles. The final concentration was 600 μM .

Trigger:

Lyophilised recombinant human tissue factor (rTF), Innovin[®] (Sysmex UK Ltd, Milton Keynes, UK) (not containing polybrene or Ca^{+2}) reagent was used as a trigger for initiation of thrombin generation and was reconstituted with 10 mL distilled water. The concentration of Innovin[®] was determined using the Actichrome[®] TF activity assay (American Diagnostica Inc., Greenwich, CT, USA) for measuring TF activity. Manufacturer's instructions were followed and several dilutions of standard and Innovin[®] were made as test samples. The standard dilutions were made from stock solution of 500 pM (available in the kit) in serial dilutions to make 30 pM, 15 pM, 7.5 pM, 3.75 pM, and 1.88 pM, and 100 μL of assay buffer as a blank. Innovin[®] sample was first diluted 1 in 10 as a stock, and then serially diluted 1:20, 1:40, and 1:80 in assay buffer. Standard dilutions and Innovin[®] sample dilutions were mixed with FVIIa and FX provided in the kit. The reagents were incubated at 37°C, allowing for the formation of TF/FVIIa complex to act on FX for conversion to FXa. The amount of FXa generated was measured by its ability to cleave Spectrozyme[®] Xa, a highly specific chromogenic substrate for factor Xa, added to the reaction solution. The cleaved substrate releases a pNA chromophore into the reaction solution and the absorbance at 405 nm was compared to those values obtained from a standard curve generated using known amounts of active human tissue factor. Reading from standard curve, the concentration of Innovin[®] was calculated as 6 μM .

The trigger for PPP TGT was prepared as follows: 10 μ L of diluted Innovin[®] (150 pM) was added to 230 μ L HWB. To this, 10 μ L of 600 μ M PL was added. The concentration of rTF and PL in this trigger were 6 pM and 24 μ M respectively. The trigger for PRP TGT was Innovin[®] diluted in HWB to give a concentration of 3 pM. Although these concentrations were recommended by Hemker *et al.* (2002) for investigation of a hypocoagulable state, a titration of TF was performed to optimise the concentration of TF.

Optimisation of trigger:

The optimal TF and PL concentration for clinical studies is still being evaluated; higher tissue factor concentrations produced less variability in the AUC and less sensitivity to contact activation, whereas lower concentrations were more sensitive to changes in thrombin generation (Dargaud *et al.*, 2010). At present, tissue factor preparations are highly variable in source material and specific activity. International standards are needed for tissue factor activators. Calibration with thrombin- α_2 -macroglobulin has the advantage that it can be used as an internal standard for samples with absorbance interference problems. As an alternative, the fluorescence signals in each experiment were normalised with a normal control throughout the study, and the results were expressed as percentage of the same normal control.

It was found that thrombin generation in the presence of corn trypsin inhibitor (CTI) and lower dose tissue factor activator seemed to be more sensitive to differences between plasma samples. Tissue factor and phospholipid concentrations were adjusted to suit specific clinical questions. In order to include the effect of FXI, it has been recommended to use a low TF concentration (Hemker, *et al.* 2002). A series of TF

(Innovin) dilution were made for optimisation of low dose TF thrombin generation. For platelet poor plasma (PPP) thrombin generation a series of 0.5 pM, 1.0 pM, 2.0 pM, 3.0 pM, 4.0 pM and 5 pM (final concentration) was made and in-house TGT performed on a normal individual PPP sample (with CTI) (Figure 2.1) and a severe haemophilia A PPP sample (with CTI) (Figure 2.2). For platelet rich plasma (PRP) TGT, first platelet count was adjusted by the individual's own PPP to obtain a platelet count of $150 \times 10^9/L$, then a series of TF dilution were made to obtain a final concentration of 0.032 pM, 0.063 pM, 0.125 pM, 0.500 pM, and 1.000 pM. In-house TGT was performed with above triggers on a sample of a normal individual (Figure 2.3) and a severe haemophilia A (Figure 2.4).

TF titration in normal control (PPP)

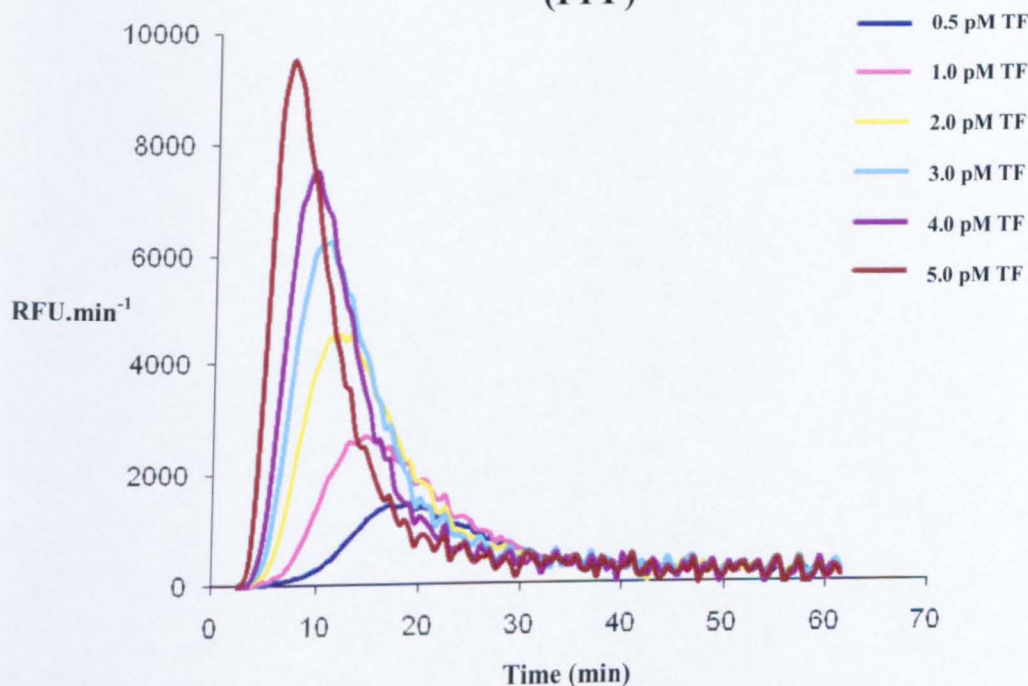


Figure 2.1 Titration of TF in a normal control (PPP)

TGT was performed on a normal control PPP sample (with CTI) to optimise the amount of TF as a low dose trigger for investigation of thrombin generation potential for a hypocoagulable state i.e. haemophilia. A series of TF dilution were made to obtain a final concentration of 0.5 pM, 1.0 pM, 2.0 pM, 3.0 pM, 4.0 pM and 5 pM, and then used as a trigger for titration of TF.

TF titration in severe HA (PPP)

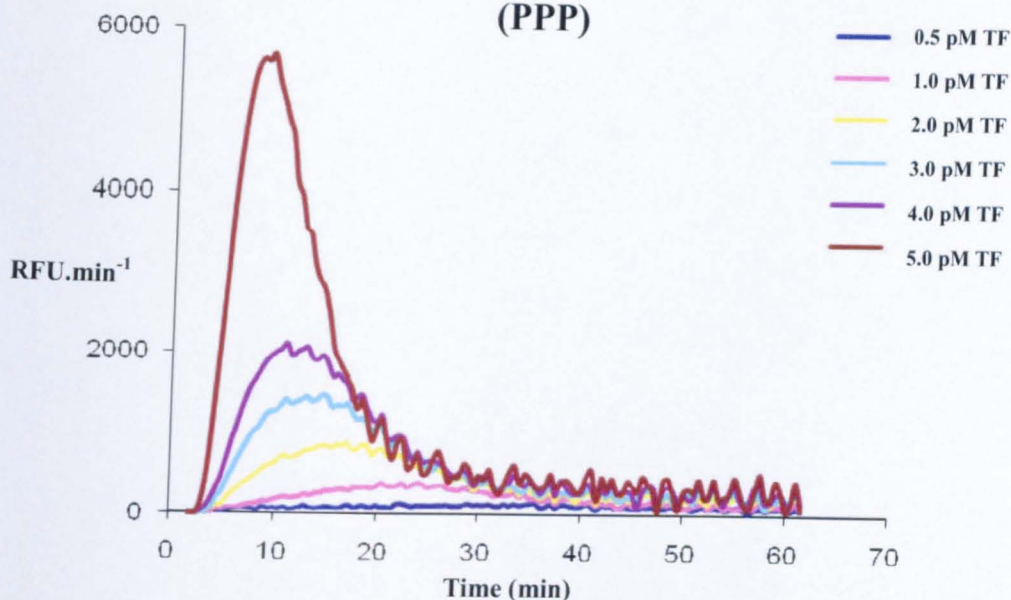


Figure 2.2 Titration of TF in a severe HA individual (PPP)

TGT was performed on a severe haemophilia A PPP sample (with CTI) to optimise the amount of TF as a low dose trigger for investigation of thrombin generation potential for a hypocoagulable state i.e. haemophilia. A series of TF dilution were made to obtain a final concentration of 0.5 pM, 1.0 pM, 2.0 pM, 3.0 pM, 4.0 pM and 5 pM, and then used as a trigger for titration of TF.

TF titration in normal control (PRP)

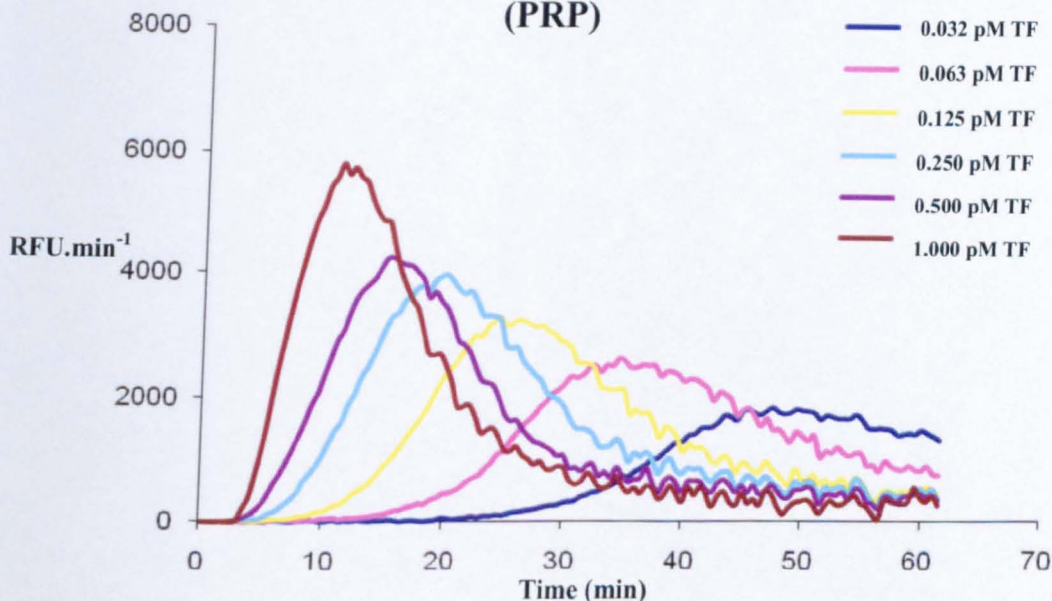


Figure 2.3 Titration of TF in a normal control (PRP)

TGT was performed on a normal control PRP sample (with CTI) to optimise the amount of TF as a low dose trigger for investigation of thrombin generation potential for a hypocoagulable state i.e. haemophilia. A series of TF dilution were made to obtain a final concentration of 0.032 pM, 0.063 pM, 0.125 pM, 0.250 pM, 0.500 pM and 1.000 pM, and then used as a trigger for titration of TF.

TF titration in severe HA (PRP)

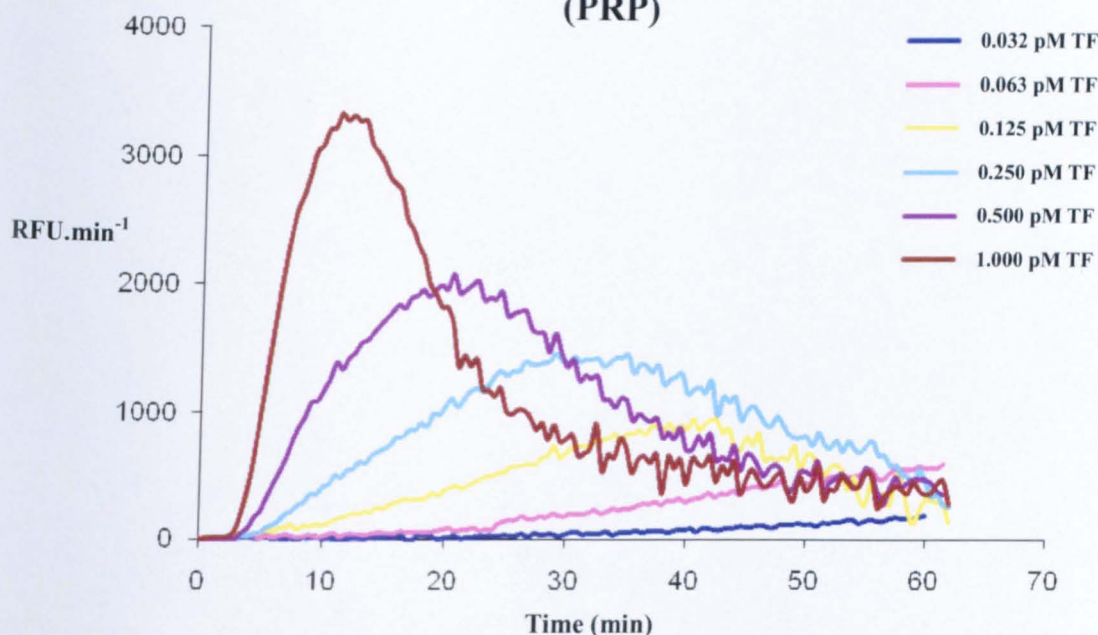


Figure 2.4 Titration of TF in a severe haemophilia A individual (PRP)

TGT was performed on a severe haemophilia A PRP sample (with CTI) to optimise the amount of TF as a low dose trigger for investigation of thrombin generation potential for a hypocoagulable state i.e. haemophilia. A series of TF dilution were made to obtain a final concentration of 0.032 pM, 0.063 pM, 0.125 pM, 0.250 pM, 0.500 pM and 1.000 pM, and then used as a trigger for titration of TF.

TGT Starting Reagent (FluCa):

A 100 mM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, St Helens, UK) was prepared by adding 0.25 g of fluorogenic substrate to 5.0 mL dimethyl sulfoxide (DMSO), aliquoted and stored at -80°C. Twenty five μ L of 100 mM DMSO fluorogenic substrate, and 100 μ L of 1.0 M CaCl_2 were added to 875 μ L HSB to make the FluCa reagent just before starting the assay. The concentrations of fluorogenic substrate and CaCl_2 were 2.5 mM and 0.1 M respectively.

Method:

Eighty μ L of test or quality control plasma (PPP/PRP), in duplicate, were added to an Immulon 2HB, round-bottom 96 well microplate (Dynex technologies, Southampton, UK). Twenty μ L of trigger (PPP/PRP) was added to each well of the plate. The final

concentration in the reaction mixture was 4.0 μ M PL and 1.0 pM rTF in PPP, and 0.5 pM rTF in PRP. The plate was then placed on a Spectramax™ Gemini XS (Molecular Devices Ltd, Wokingham, UK) spectrofluorometer, and shaken for a few seconds, then incubated for 2 min at 37°C. Twenty μ L of FluCa was added immediately to each wells and shaken promptly, before starting the spectrofluorometer. The plate was read at 30 s intervals for one hour, the fluorochrome of the fluorogenic substrate excited at 390 nm wavelength, and then emitted light at 460 nm wavelength. The emitted light was detected and plotted using SOFTmax PRO software (Molecular Devices Ltd, Wokingham, UK) provided by manufacturer.

Data Analysis:

The data were exported into an EXCEL® file and the amount of thrombin generated was calculated according to the method by Hemker (Hemker, 2005), and modified (see below) by the National Institute for Biological Standards and Control (NIBSC). The spreadsheet was kindly donated by Dr. Sanj Raut (NIBSC).

This software was used to determine the amount of free thrombin in a continuous TGT with a fluorogenic substrate, by subtracting thrombin- α_2 -M complex. α_2 -M is a large plasma protein, which inhibits the coagulation system by binding to thrombin, and the fibrinolysis system by inactivating plasmin. The thrombin in the α_2 -M-IIa complex has no known biological activity but *in vitro* retains its ability to cleave fluorogenic substrate in test experiments. Therefore it is necessary to subtract from the total thrombin generated in the experiment. The EXCEL® spreadsheet with enabled MACRO formulas enabled us to calculate free thrombin from relative fluorogenic unit (RFU) produced by cleavage of substrate (Table 2.1). From free-thrombin data the

peak-height (PH) and time to peak-height (TP) were calculated (Table 2.2). In addition the area under the thrombin generation curve (AUC) was calculated by the trapezium rule. The amount of thrombin generated was calculated according to the method by Hemker (Hemker & Beguin, 1999) (Table 2.1, Figure 2.1). This method was used by Hemker *et al.* to determine the amount of free thrombin in a continuous TGT with a fluorogenic substrate (Hemker *et al.*, 2000).

Table 2.1 Calculation of free thrombin

A	B	C	D	E	F
1	Time (min)	Data (total thrombin)	Product from free thrombin	Product from α_2 -M	k
2	0.5		=C3-E2	=E2+(\$F\$1*D3)	=sum(F112:F122)
3	1		=C4-E3	=E3+(\$F\$1*D4)	=(D4-D3)/0.5
4	1.5		=C5-E4	=E4+(\$F\$1*D5)	=(D5-D4)/0.5

Free thrombin was calculated on an excel[®] spreadsheet by transferring the raw data (fluorogenic relative unit (RFU)), which was the total thrombin generated, into the column C. The total thrombin RFU was measured every 30 s (0.5 min) in column B. In column E the amount of thrombin- α_2 -M complex was calculated by using of a k which was derived from the last 10 measurement of the reaction.

A macro was established to calculate peak height and time to peak (Table 2.2) according to the method by Hemker *et al.* (2000).

Table 2.2 Calculation of peak height and time to peak thrombin generation

	A	B	C	D
1		1	2	3
2	TP	=INDEX(\$A\$6:\$A\$125,B3,1)	=INDEX(\$A\$6:\$A\$125,C3,1)	=INDEX(\$A\$6:\$A\$125,D3,1)
3		=MATCH(B4,B6:B125,0)	=MATCH(C4,C6:C125,0)	=MATCH(D4,D6:D125,0)
4	PH	=MAX(B6:B125)	=MAX(C6:C125)	=MAX(D6:D125)
5		1	2	3
6	1	Data	Data	Data
7	1.5	Data	Data	Data

Equations for the calculation of peak height and time to peak from free thrombin values. Using a macro enabled formula on EXCEL[®] spreadsheet peak height (PH) and time to peak (TP) thrombin generation were calculated.

The fluorogenic substrate was titrated to investigate if the rate and total amount of thrombin generation is optimal at the concentration 2.5 mM which was recommended by Hemker *et al.* (2002) (Figure 2.5)

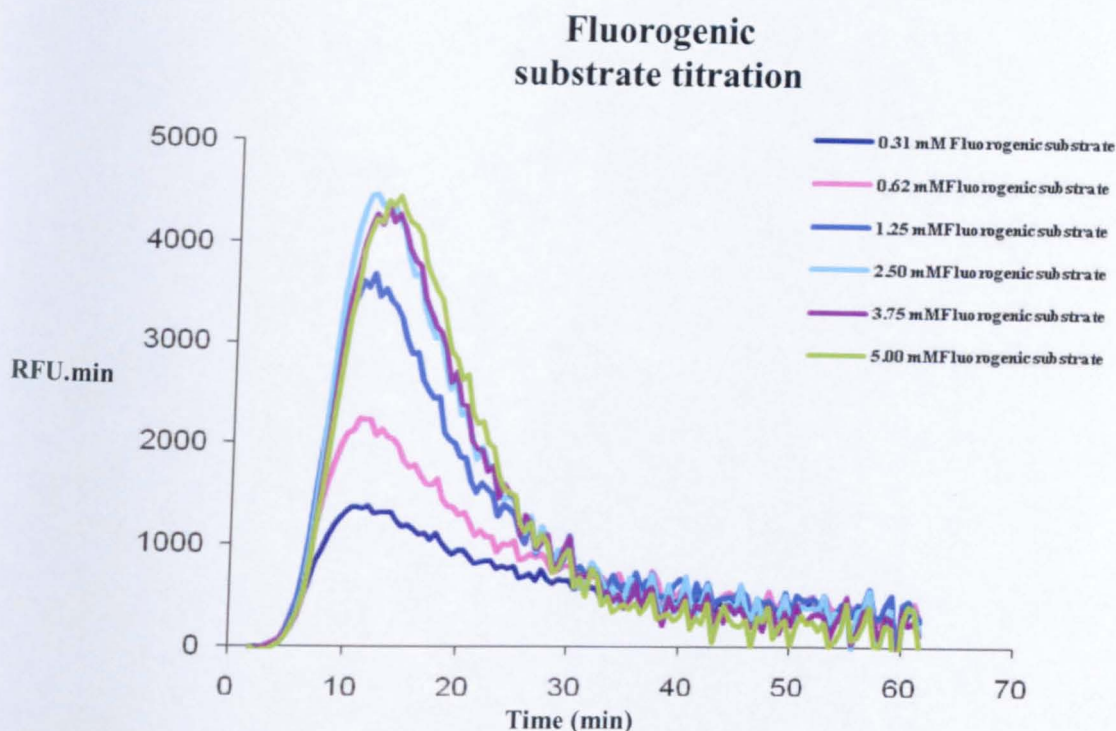


Figure 2.5 Titration of fluorogenic substrate in a normal control

Fluorogenic substrate was titrated in a normal control (PPP with CTI). An increase in peak height thrombin generation was noticed up to concentration of 2.5 mM (final concentration). The increase above this level did not change the rate or the total amount of thrombin generation.

In this method a normal control was used throughout of the study, and in each experiment, to enable us to transform the raw data to the percentage of the normal. However, the concentration of free thrombin could not be calculated in this method. In order to calculate the concentration of free thrombin, an appropriate calibration should have been used to correct for inner-filter effects, i.e. the quenching of the fluorescence signal by substrate molecules that have already been converted, and the

effect of substrate consumption. This was realised by Hemker *et al.* and they introduced the α_2 -M-IIa complex as a calibrator in the CAT system (Hemker *et al.*, 2003). This study was commenced before the CAT was commercialised, and once the KDHCTU purchased the analyser, thrombin generation was performed on frozen aliquots of PPP samples which were saved at the time of processing, and the results were compared with the in-house TGT method as it is reported in chapter 4.

2.4.9. Calibrated Automated Thrombogram[®] (CAT)

The calibrated automated thrombography[®] (CAT) (Hemker *et al.*, 2003) instrument (Diagnostica Stago Ltd, Reading, UK), was used to analyse the quality controls and test samples along with the in-house TGT. The CAT package included:

- Thrombin calibrator: Thrombin calibrator was reconstituted in 1.0 mL distilled water.
- PPP-Reagent Low: Used as a trigger and was added to PPP to initiate thrombin generation. PPP-Reagent Low contains a mixture of 4.0 μ M PL and 1.0 pM rTF (the concentration is the final concentration after adding plasma and FluCa).
- FluCa Kit: Fluo-buffer which contains HEPES buffer and CaCl₂, and Fluo-substrate contains the fluorogenic substrate solubilised in dimethyl sulfoxide (DMSO).

Method

Using Immulon 2HB, round-bottom 96-well plates (Dynex technologies), each experiment needed two sets of readings, one from a well in which thrombin

generation was taking place (TG well) and a second one from a well to which the calibrator was added (CAL well). The experiments were carried out in duplicate, i.e. a set of two TG wells were compared to a set of two CAL wells. To each well, 80 μ L of plasma was added. The TG wells receive 20 μ L of the trigger, and the CAL wells 20 μ L of the α_2 -M-T solution. At the start of the experiment, the instrument dispenses 20 μ L of FluCa to all the wells to be measured, registers this as zero time, shakes them for 10 s and starts reading with a 390/460 filter set (excitation/emission). During the measurement, the thrombinoscope[®] software uses a default calibrator to calculate tentative (before excluding the α_2 -M-T) thrombin concentrations and displays the course of thrombin concentration in time for each group. After the measurement, the program calculates all parameters of the CAT including: endogenous thrombin potential (ETP), peak height, lag time, time to peak and expresses the results in nanomolar thrombin over time.

Standardisation of results:

In order to compare the results produced by two methods, a normal control was used throughout study and in each experiment; the results were calculated as percentage of the normal control in each run.

2.4.10. Rotational Thromboelastometry (ROTEM[®])

Using the non-activated thromboelastometry (NATEM) protocol as a guide ROTEM[®] (Pentapharm GmbH, Munich, Germany), ROTEM[®] was performed on citrated whole blood (CWB) samples with or without CTI and rTF. rTF stock was diluted 1 in 1000 (20 mM Hepes, 140 mM NaCl, pH 7.35 dilution buffer) to make a solution of 6.0 pM rTF. Where the test was performed with rTF, 20 μ L of diluted rTF was added to the

ROTEM[®] cup, immediately followed by 300 μ L test or quality control samples (with/without CTI) followed by 20 μ L of 200 mM CaCl₂, mixed and immediately returned to the ROTEM[®] instrument, to perform the test. The tests were halted after two hours and the following parameters CT, CFT, α° , and MCF were extracted from the analyser. The curves were subsequently analysed using the software program provided with the ROTEM[®] analyser and raw data was imported into an EXCEL[®] worksheet for analysis of MaxVel, tMaxVel, and AUC parameters.

2.4.11. Clot waveform analysis

The APTT test was performed on the MDA[®] coagulometer (Organon Teknika, Cambridge, UK) using MDA[®] reagents Platelin LS and Platelin LS CaCl₂ (Organon Teknika), in the Haemostasis laboratory, Addenbrooke's Hospital, Cambridge, UK with kind permission of Dr Roger Luddington. A frozen aliquot (stored at -80°C immediately after processing) of patients and normal control group were taken on dry ice to Addenbrooke's Hospital for testing.

The MDA[®] measured continuous changes in light transmittance that occur as the test/quality control plasma sample clotted. The waveform was mathematically processed by a software algorithm, provided with the MDA[®], to derive several parameters such as coagulation velocity (Min1) by measuring the percentage difference in light transmittance divided by time difference in seconds (%T/s), and acceleration (Min2) was calculated based on difference in percentage of change in light transmittance over time in squared seconds (%T/s²) (Shima *et al.*, 2002).

Chapter 3 ROTEM[®] analysis

3.1. Introduction

The majority of coagulation tests only detect the initiation phase of clotting *in vitro* in plasma following addition of excessive amounts of activating reagent and unphysiological phospholipids to substitute the missing cell surfaces and platelets. However, ninety five percent of the thrombin generation during the coagulation process, *in vivo*, takes place after the initiation phase of the clot formation (Abrahams *et al.*, 2002; Baglin, 2005; Mann, 2003). Also several studies have demonstrated the importance of the functions of platelets (Yee, 2006) and leukocytes during coagulation (Bouchard & Tracy, 2001; McEver, 2001). Thus whole blood coagulation analysis, to assess global haemostasis, theoretically appears more informative compared to plasma based assays such as the most common laboratory screening tests- PT and APTT, although the contribution of vessel endothelium is necessarily excluded.

Rotational thromboelastometry (ROTEM[®]) has been used for many years to assess the overall haemostatic function in whole blood, by evaluating the changes in the viscoelastic properties of blood during coagulation, and captures the information on the interaction of platelets and blood cells with the proteins of the coagulation process from the time of the initial platelet-fibrin interaction, through platelet aggregation, fibrin cross linkage, to eventual clot lysis. With the advent of computer-assisted equipment, and a modification, originally described by Sørensen *et al.* (2003), interest has resurged in this technique. Sørensen *et al.* modified the ROTEM[®] analysis by initiating whole blood clot formation with a small amount of TF in order to mimic the

in vivo coagulation process in the ROTEM[®] cup. They mathematically extracted the first derivative of ROTEM[®] tracing by CalcuRo[™] software and introduced three new parameters, maximum velocity (MaxVel), time to maximum velocity (tMaxVel), and area under the velocity curve (AUC) which may add more information in assessing the haemostasis status of individuals.

However, to exclusively investigate TF-dependent coagulation and clot formation, and mimic the *in vivo* haemostasis scenario, the contact pathway must be eliminated in order to diminish possible artificial activation which could be triggered by venepuncture, sample handling or other pre-analytical variables. Corn trypsin inhibitor (CTI) has been used to quench the contact pathway by inhibiting FXIIa (Rand *et al.*, 1996); (Dargaud *et al.*, 2006b) in thrombin generation based on Hemker's (2005) method. The use of CTI in a thrombin generation assay has been studied by different groups, and it has been shown, when low dose of TF is used to trigger coagulation, CTI reduces the variability of thrombin generation within- and between-individuals (Dargaud *et al.*, 2006b; Luddington & Baglin, 2004). In another study utilising ROTEM[®] to monitor the effect of FVIII replacement therapy, (Lewis *et al.*, 2007), CTI was used to quench contact activation, however, but to this date there has been no systematic study looking into the effects of CTI on low dose tissue factor triggered whole blood coagulation as assessed by ROTEM[®].

3.2. Aims

The aim of this study was to evaluate the usefulness of ROTEM[®] in diagnosing haemophilia, when performed by three different techniques. The techniques include

- i. Activation of blood coagulation through the contact pathway
- ii. Activation of blood coagulation through the extrinsic pathway with the use of TF
- iii. Activation of blood coagulation through the extrinsic pathway with the use of TF, where contact activation has been inhibited by CTI.

3.3. Study subjects

Ninety seven patients were consented for the study, and ROTEM[®] data for further analysis was available for 70 patients. This included 30 severe HA, 11 moderate HA, 16 mild HA, seven severe HB, three moderate HB, and three mild HB. The age range was between 18 and 90 years (mean 43, median 41 years). The control group comprised 22 healthy male individuals aged between 22 and 62 (mean 40, median 39 years). All severe haemophilia samples included in the statistical analysis had FVIII or FIX activity levels of < 1.0 IU/dL, as measured by the one stage clotting assay.

3.4. ROTEM[®] analysis

Citrated whole blood (CWB) was analysed by ROTEM[®], as was described in section 2.4.10, to assess the global haemostasis profile in three test categories:

- i. CWB samples in which coagulation was started by addition of CaCl₂ (activated by the surface of the measurement cup).
- ii. CWB samples in which TF (0.35 pM final concentration) was added before the start of the final reaction by CaCl₂, denoted CWB+TF.

- iii. CWB samples in which CTI was added to the sample tube prior to blood collection, then initiated with TF (0.35 pM final concentration) just before the final reaction started by CaCl_2 . Denoted CWB+CTI+TF.

3.4.1. Conventional ROTEM[®] parameters

The following conventional parameters were analysed in this study

- Clotting time (CT)
- Clot formation time (CFT)
- Alpha angle (α°).

3.4.2. Derived ROTEM[®] parameters

The velocity profile of thromboelastometry was processed using CalcuRo[™] software, for calculation of dynamic coagulation parameters (Figure 1.9c).

- Maximum velocity (MaxVel): describes the maximum rate of clot formation and is calculated from the 1st derivative of the ROTEM[®] tracing curve. This is dependent on the activity of intrinsic clotting factors including FVIIIa, FIXa, FXIa, FXIIa, prothrombinase complex, and fibrinogen level. Where CTI was used and the coagulation was initiated by small amount of TF, the MaxVel would be depended upon the activities of FVIIa, FVIIIa, FIXa, FXIa, prothrombinase complex, and fibrinogen level.
- Time to maximum velocity (tMaxVel): measures the time from the start of the reaction until the maximum velocity. This is related to clotting factors, fibrinogen and the platelet count.

- Area under the curve (AUC): this is the area under the velocity curve, i.e. the area under the first derivative curve, and it quantifies the maximum clot firmness.

3.5. Data and statistical analysis

Conventional and derived parameters were analysed for controls and all patient groups (severe, moderate, and mild HA; severe, moderate, and mild HB) in three test categories (CWB, CWB+TF, and CWB+CTI+TF).

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA). Mann-Whitney *U* test was performed to compare the parameters between the different patient groups and the controls. Wilcoxon rank test was performed to compare paired parameters across the test categories, and Kruskal-Wallis nonparametric ANOVA test was performed to analyse the variance between the three test categories. The correlation coefficient was determined using the Spearman rank method. Receiver operating characteristic curve (ROC) was performed to evaluate the sensitivity and specificity of the ROTEM[®] analysis. In all statistical analysis, a *p* value of < 0.05 was considered significant (Zweig & Campbell, 1993)

3.6. Results

3.6.1. ROTEM® conventional parameters

The results showed a nonparametric distribution for the majority of ROTEM® conventional parameters (CT, CFT, and α°), therefore median and the range (based on 5th- 95th percentiles) was calculated for each parameter (Table 3.1). Intra assay variation (based on four simultaneous measurements of two normal male subjects) was CT = 6.8%, CFT = 9.0%, α° = 4.9%, AUC = 9.3%, MaxVel = 5.9% and tMaxVel = 8.3%. Inter-assay variation (based on four simultaneous measurements of two normal male subjects) was CT = 9.8%, CFT = 13.8%, α° = 15.4%, AUC = 9.8%, MaxVel = 11.7% and tMaxVel = 11.1%.

Table 3.1 ROTEM® conventional parameters

ROTEM® Parameters	Test Category	Normal <i>n</i> = 22 Median (5 th - 95 th percentile)	Severe HA <i>n</i> = 30 Median (5 th - 95 th percentile)	Moderate HA <i>n</i> = 11 Median (5 th - 95 th percentile)	Mild HA <i>n</i> = 16 Median (5 th - 95 th percentile)	Severe HB <i>n</i> = 7 Median (5 th - 95 th percentile)	Moderate HB <i>n</i> = 3* Median (5 th - 95 th percentile)	Mild HB <i>n</i> = 3* Median (5 th - 95 th percentile)
CT _s	CWB	597 (354-937)	1783 (1132-5524)	1262 (772-1580)	1019 (641-1328)	2020.5 (1156-3206)	1202 (1052-1306)	890 (687-1092)
	CWB+ TF	311 (186-437)	754 (372-1779)	486 (348-757)	483 (288-1398)	771.5 (489-1333)	567 (374-762)	575 (345-805)
	CWB+CTI +TF	412.5 (201-612)	1018 (464-2832)	768.5 (374-1652)	727 (409-1554)	859 (497-3678)	677 (311-1043)	583 (431-764)
CFT _s	CWB	242.5 (150-473)	536.5 (288-1434)	378.5 (284-522)	355 (177-445)	819 (302-1255)	477 (270-660)	400 (278-521)
	CWB+ TF	122 (95-226)	291 (180-587)	211 (173-326)	211 (102-323)	391 (165-470)	258 (194-374)	278 (200-365)
	CWB+CTI +TF	177.5 (112-280)	544 (256-1773)	413 (211-524)	366 (183-738)	594 (261-922)	339 (244-568)	263 (244-286)
α°	CWB	49.5 (30.4-61.9)	28.5 (14.4-44.6)	36.5 (28-44)	37 (26-57)	20 (10-44)	32.7 (23-46)	36 (28-44)
	CWB+ TF	64 (51.0-71.0)	45 (26.5-57)	53 (40-58)	53 (42-69)	35 (31-59)	48.3 (36-56)	45.5 (37-54)
	CWB+CTI +TF	57.5 (47.0-67.7)	27.5 (10.9-50.6)	35 (28-52)	39 (26-58)	25 (17-47)	44.5 (26-56)	50.7 (44-56)

The median and the range (5th- 95th percentiles) ROTEM® conventional parameters were calculated in control group and six patient groups in three test categories. CT = clotting time, CFT = clot formation time, HA = haemophilia A, HB = haemophilia B, CWB = citrated whole blood, TF = tissue factor, CTI = corn trypsin inhibitor, α° = alpha angle.
*where the number of samples was not statistically significant, minimum and maximum values were reported

3.6.1.1. Clotting time

CT was analysed for controls, patient groups, and the test categories. The reference ranges were established using the control group (Table 3.1). A significant difference ($p = 0.007$) was found between the median of control and patient groups in all test categories (Figures 3.1, 3.2, and 3.3).

No significant difference was found in the CT between moderate and mild HA in any of the test categories. A significant difference was noted when the severe and moderate HA groups were compared only in the CWB test category ($p = 0.0019$) showing that CT in CWB test category may differentiate between a severe and a moderate haemophilic patient. In HB groups, a significant difference was found between mild HB and the control group ($p = 0.007$), and between severe and moderate HB ($p = 0.033$), but no significant difference was found between mild and moderate HB ($p = 0.998$).

The coefficient of variation (CV) was also calculated for all the groups and three test categories, which revealed a wide variation. The CV was highest for the CWB+CTI+TF test category, (severe HA (61%), moderate HA (51%), and mild HA (62%)) when compared to the CWB+TF (47, 30, and 50% respectively), and the CWB (53, 24, and 16% respectively). These results show that the use of CTI might potentially identify the heterogeneity amongst the patients in the same group.

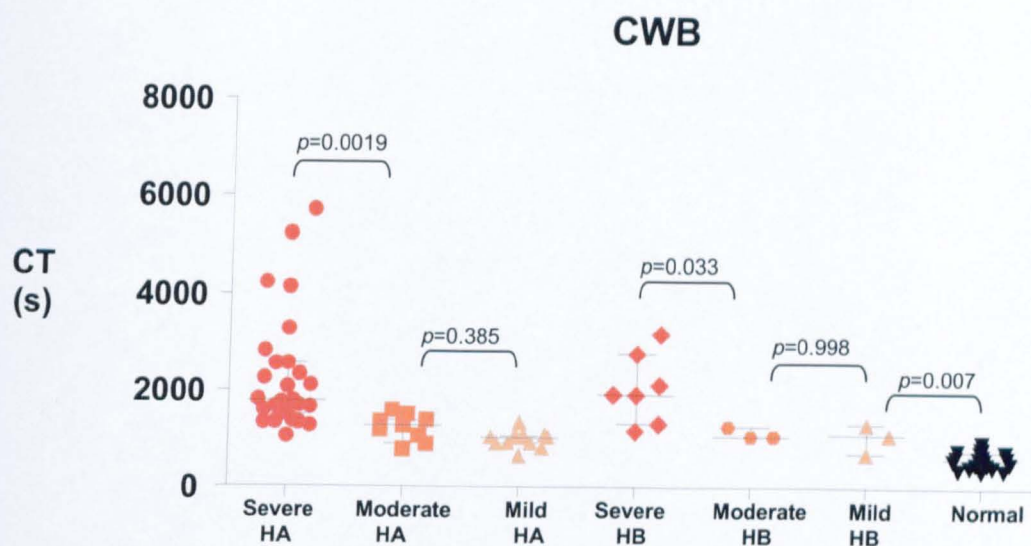


Figure 3.1 ROTEM® CT parameter in CWB

Severe, moderate, mild HA and HB compared to control group in CWB category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

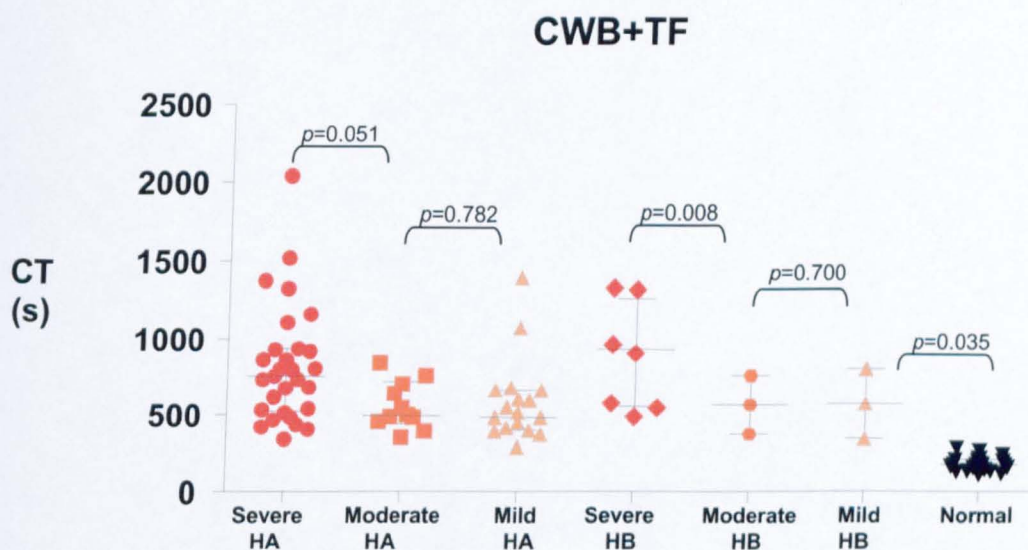


Figure 3.2 ROTEM® CT parameter in CWB+TF

Severe, moderate, mild HA and HB compared to control group in CWB+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

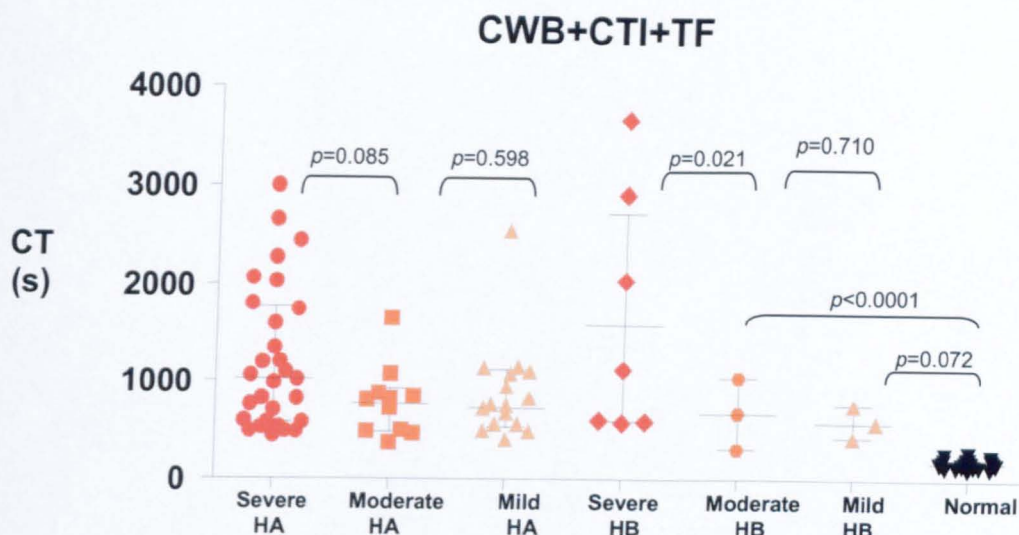


Figure 3.3 ROTEM® CT parameter in CWB+CTI+TF

Severe, moderate, mild HA and HB compared to control group in CWB+CTI+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

CT in the severe HA group was compared between all test categories by using the Wilcoxon rank test; and showed significant differences between the three test categories (CWB, CWB+TF, and CWB+CTI+TF) ($p < 0.0001$).

3.6.1.2. Clot formation time

The median and 5th- 95th percentile range for CFT were calculated (Table 3.1) and compared to the control group (Figures 3.4, 3.5, and 3.6). A significant difference in prolongation of the CFT was shown by the Mann Whitney U test between the control group and six patient groups in the three test categories (the highest $p = 0.031$ between mild HA and the control group in CWB test category). There was a significant difference between severe HA and moderate HA in CWB+CTI+TF ($p = 0.021$) and CWB ($p = 0.022$) test categories but not in CWB+TF ($p = 0.130$) category.

The difference between moderate and mild HA, however, was not significant in any test category. This may indicate the test is not sensitive enough to distinguish between the two groups, or the current classification of severities, based on the coagulation factor levels, may not reflect the overall clot formation potential of an individual.

The CV of CFT was highest in CWB+CTI+TF test in severe HA (63%), moderate HA (29%), and mild HA (38%) when compared to CWB+TF (36, 27, and 28% respectively), and CWB (50, 21, and 27% respectively). In HB groups, a wide variation was noticed among severe HB, which was similar to the severe HA group.

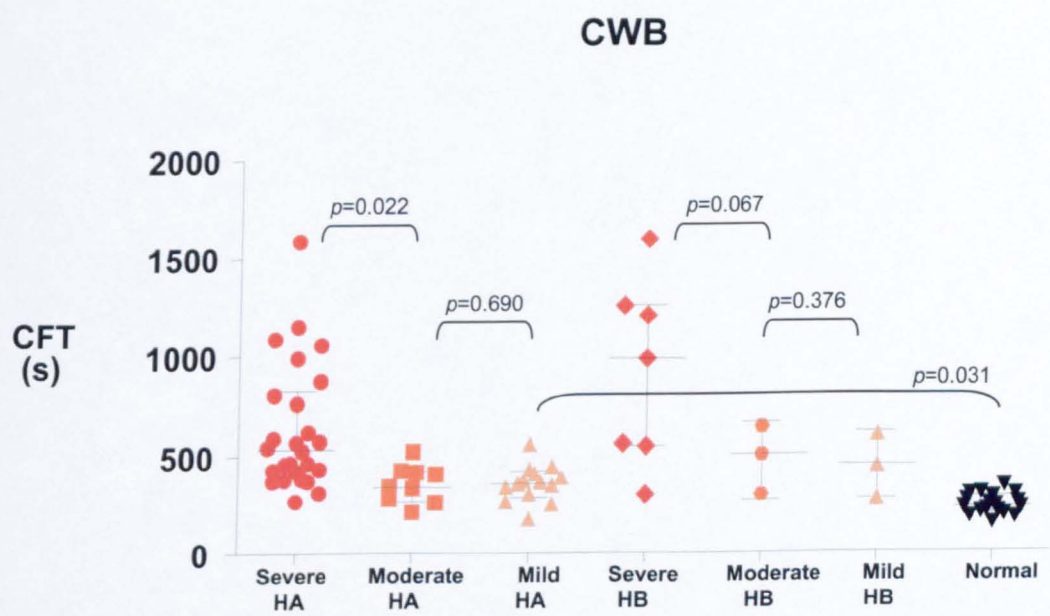


Figure 3.4 ROTEM® CFT parameter in CWB
Severe, moderate, mild HA and HB compared to the control group in CWB category. Error bars show the median and inter-quartile range. (the *p* values are for the indicated paired populations)

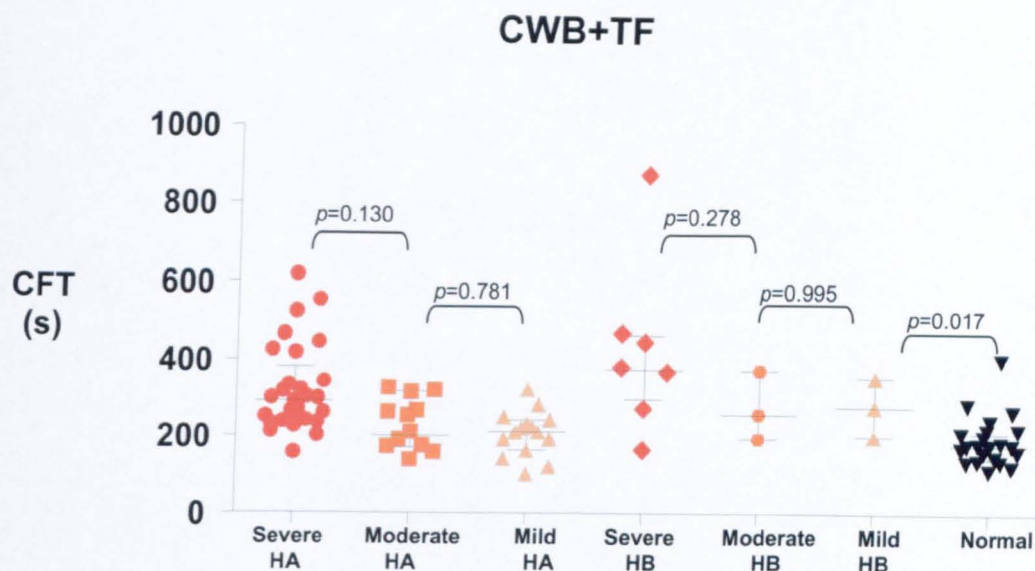


Figure 3.5 ROTEM® CFT parameter in CWB+TF
 Severe, moderate, mild HA and HB compared to the control group in CWB+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

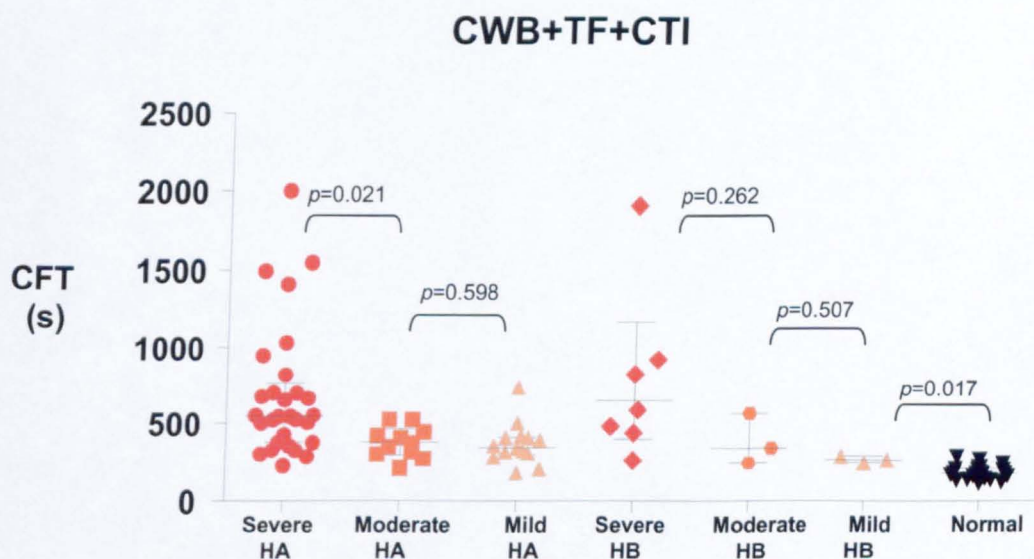


Figure 3.6. ROTEM® CFT parameter in CWB+CTI+TF
 Severe, moderate, mild HA and HB compared to the control group in CWB+CTI+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

3.6.1.3. Alpha angle

The median and 5th- 95th percentile range for α° were calculated (Table 3.1) and compared to the control group (Figures 3.7, 3.8, and 3.9). A significant decrease in α° was shown by the Mann-Whitney *U* test between the control group and the six patient groups in the three test categories ($p < 0.05$). There was no significant difference found between severe and moderate HA in CWB+CTI+TF ($p = 0.057$) and CWB+TF ($p = 0.116$) test categories, however, there was a significant difference in CWB test category ($p = 0.0401$). There was no significant difference in α° between moderate and mild HA ($p = 0.446$ in CWB+CTI+TF, $p = 0.258$ in CWB+TF, and $p = 0.629$ in CWB). In HB groups, a significant difference was found between the mild HB and the control group ($p = 0.028$) but no significant difference between mild and moderate ($p = 1.0$), or moderate and severe ($p = 0.183$) HB was found.

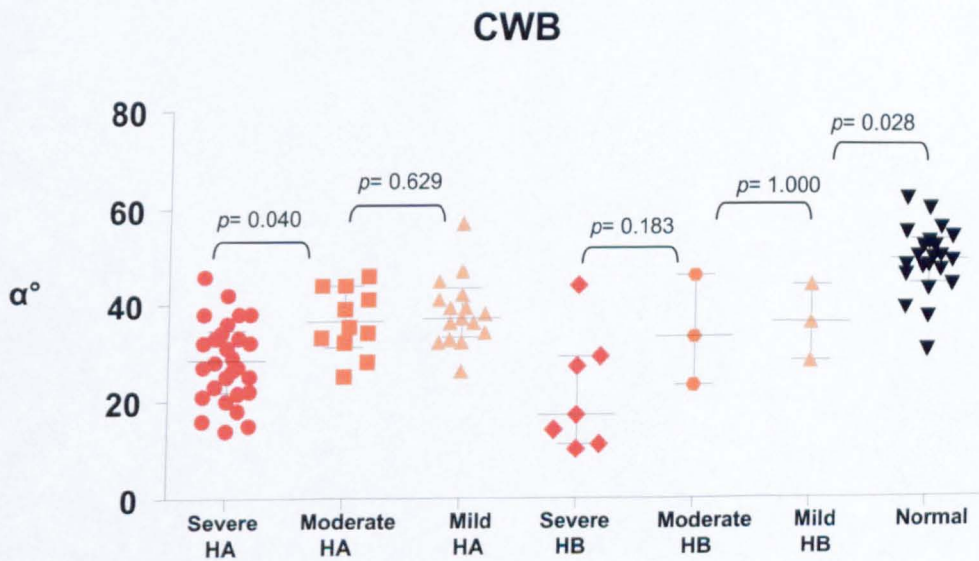


Figure 3.7 ROTEM[®] alpha angle parameter in CWB
Severe, moderate, mild HA and HB compared to the control group in CWB category. Error bars show the median and inter-quartile range. (the *p* values are for the indicated paired populations)

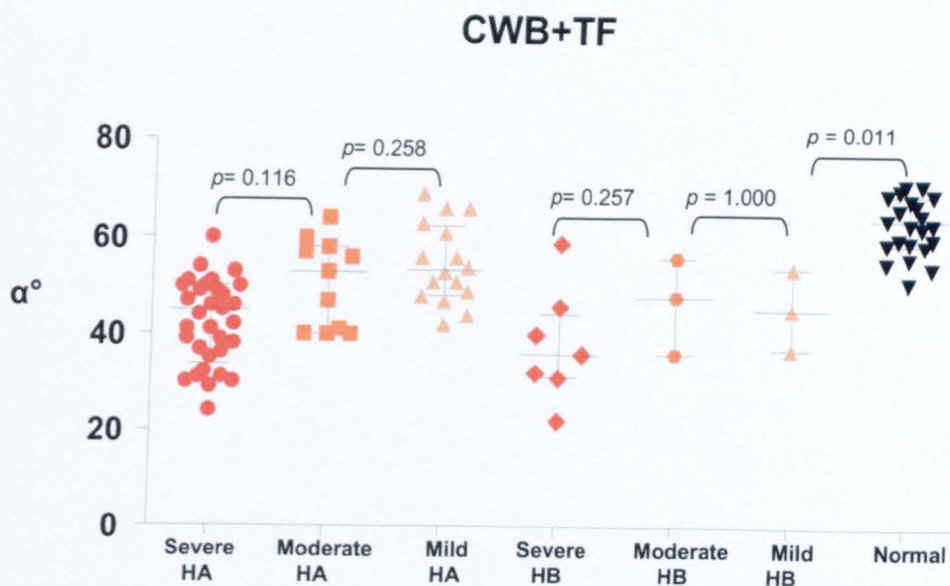


Figure 3.8 ROTEM[®] alpha angle parameter in CWB+TF
 Severe, moderate, mild HA and HB compared to the control group in CWB+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

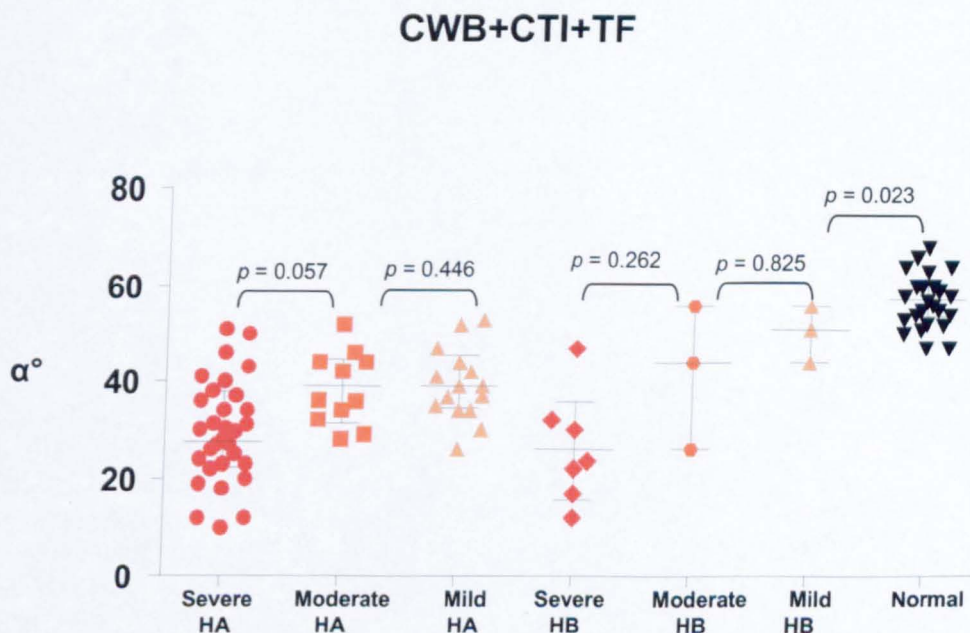


Figure 3.9. ROTEM[®] alpha angle parameter in CWB+CTI+TF
 Severe, moderate, mild HA and HB compared to the control group in CWB+CTI+TF category. Error bars show the median and inter-quartile range. (the p values are for the indicated paired populations)

3.6.2. ROTEM® derived parameters

The ROTEM® derived parameters (MaxVel, tMaxVel, and AUC) for the control group and the all patient groups in three test categories were analysed (Table 3.2). The results showed a nonparametric distribution for the majority of the parameters, therefore median and the range (based on 5th- 95th percentiles) was calculated for each parameter. Intra assay variation (based on four simultaneous measurements of two normal male subjects) was AUC = 10.2%, MaxVel = 7.1% and tMaxVel = 8.3%. Inter-assay variation (based on measurements of two normal male subjects over four different days) was AUC = 12.8%, MaxVel = 10.5% and tMaxVel = 10.3%.

Table 3.2 ROTEM® derived parameters

ROTEM® Parameters	Test Category	Normal <i>n</i> = 22 Median (5 th - 95 th percentile)	Severe HA <i>n</i> = 30 Median (5 th - 95 th percentile)	Moderate HA <i>n</i> = 11 Median (5 th - 95 th percentile)	Mild HA <i>n</i> = 16 Median (5 th - 95 th percentile)	Severe HB <i>n</i> = 7 Median (5 th - 95 th percentile)	Moderate HB <i>n</i> = 3* Median (5 th - 95 th percentile)	Mild HB <i>n</i> = 3* Median (5 th - 95 th percentile)
MaxVel mm.min ⁻¹	CWB	7.4 (5.1-12.5)	3 (1.2-5.2)	3.3 (2.0-4.0)	4 (3.0-8.0)	2 (2.0-5.0)	3.3 (2.0-4.8)	4 (3.0-5.0)
	CWB+ TF	10 (7.0-15.2)	5 (2.6-8.3)	5 (3.4-8.3)	7 (5.0-12.0)	3.5 (3.0-9.0)	5.7 (4.0-7.0)	6.5 (6.0-7.0)
	CWB+CTI +TF	8.95 (6.5-13.7)	3.0 (1.9-6.3)	3.5 (2.90-4.9)	5 (3.0-8.0)	3 (2.0-6.0)	5.2 (2.5-7.8)	7 (5.0-7.0)
tMaxVel min	CWB	9 (5.5-13.5)	38.7 (21.9-92.0)	25.9 (18.3-33.0)	19.7 (13.3-26.8)	46.3 (24.3-68.6)	23.9 (20.6-26.2)	15.9 (11.7-20.5)
	CWB+ TF	7.7 (3.5-12.3)	16.9 (9.8-35.4)	12.4 (10.8-18.9)	10.5 (6.6-13.9)	17.6 (11.1-29.6)	11.5 (8.4-14.3)	11.2 (7.9-14.6)
	CWB+CTI +TF	8.4 (4.7-14.9)	22.6 (9.3-52.2)	19.2 (12.5-32.2)	15.8 (10.1-26.9)	23.3 (11.3-48.0)	16.1 (10.8-22.3)	11.6 (7.7-15.4)
AUC mm	CWB	55 (43.6-60.0)	47.6 (27.8-61.0)	44.2 (39.7-58.7)	48.1 (35.4-60.0)	46.9 (36.1-55.1)	40.5 (34.2-48.7)	43.5 (39.9-46.5)
	CWB+ TF	58.8 (44.1-82.0)	51.6 (39.2-69.5)	49.1 (43.0-63.4)	53.6 (46.8-62.6)	52.3 (43.0-57.0)	51.2 (44.0-56.1)	50.1 (47.2-53.0)
	CWB+CTI +TF	55.5 (48.4-62.3)	48 (40.5-68.3)	51.6 (44.6-65.0)	52.5 (42.8-62.0)	47.1 (40.4-53.1)	55.4 (42.9-67.8)	52.5 (49.7-54.4)

The median and the range (5th- 95th percentiles) ROTEM® derived parameters were calculated in control group and Haemophilia patients in three categories. MaxVel = maximum velocity, tMaxVel = time to maximum velocity, AUC = area under the first derivative curve, HA = haemophilia A, HB = haemophilia B, CWB = citrated whole blood, TF = tissue factor, CTI = corn trypsin inhibitor.

*where the number of samples was not statistically significant, minimum and maximum values were reported

3.6.2.1. Maximum velocity

Maximum velocity (MaxVel) parameter of ROTEM[®] was analysed in all the patient groups and the median and range calculated (Table 3.2). The Mann-Whitney *U* test showed the MaxVel in severe, moderate, mild HA and HB was significantly lower in all test categories when compared to the control group (the highest $p = 0.0405$ between moderate HB and the control group (Figure 3.12)). However, the Mann-Whitney *U* test was not significant when MaxVel was compared between the severe and the moderate HA groups ($p = 0.148$) in CWB+CTI+TF, CWB+TF ($p = 0.249$), and CWB ($p = 0.981$) test categories, although a significant difference was found between the MaxVel of moderate and mild HA in CWB+CTI+TF ($p = 0.019$) and CWB ($p = 0.008$) test categories, but not in CWB+TF test category ($p = 0.097$).

Wilcoxon rank test was performed to investigate if MaxVel is different between test categories. The results showed a significant difference between CWB+TF and CWB+CTI+TF ($p = 0.0004$) where median MaxVel was higher in CWB+TF. This indicates that the addition of TF, without exclusion of contact activation by CTI, may cause overestimation of the results. There was no significant difference between MaxVel in CWB and CWB+CTI+TF ($p = 0.529$). Comparing the difference between the MaxVel in CWB and CWB+TF, a significant was found ($p = 0.0005$). This may suggest that the initiation, whether through contact or TF, may return the same results.

In HB groups, no significant difference was found between severe, moderate, and mild patient groups. This may be due to the small numbers in each group. The difference between groups in different categories has been illustrated in Figures 3.10, 3.11, and 3.12.

The CV for the MaxVel parameter in the severe, moderate and mild HA in CWB+CTI+TF was 41%, 27%, and 26% respectively. These findings are consistent with other studies which reported a large heterogeneity of ROTEM[®] parameters (Ingerslev *et al.*, 2003; Sørensen & Ingerslev, 2004). In the total HB group ($n = 13$) MaxVel was significantly different between the HB group and the control group ($p < 0.0001$).

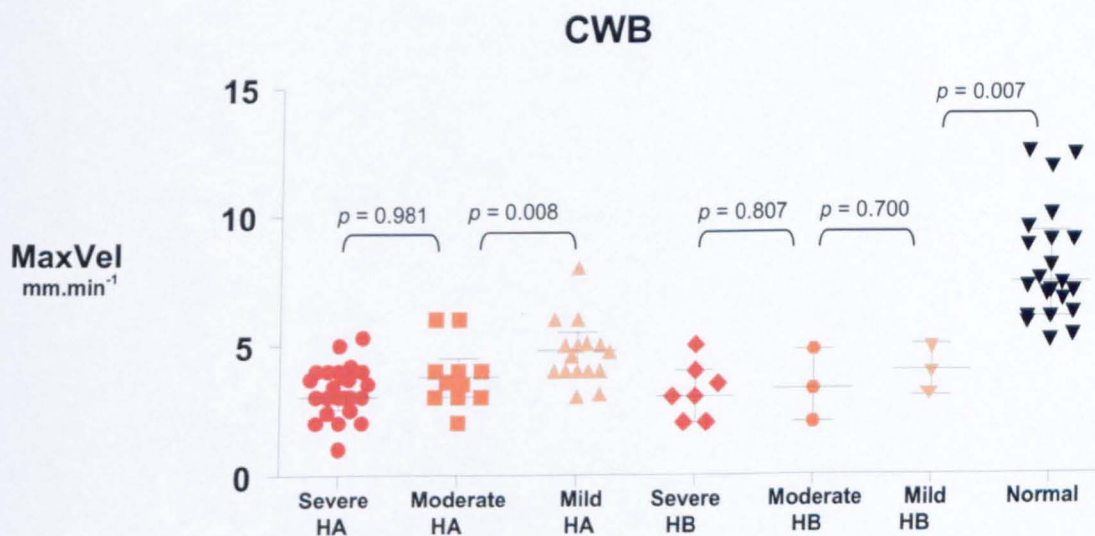


Figure 3.10 ROTEM[®] MaxVel comparison in CWB

The graph depicts MaxVel parameter in severe, moderate, mild HA and HB patients and the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

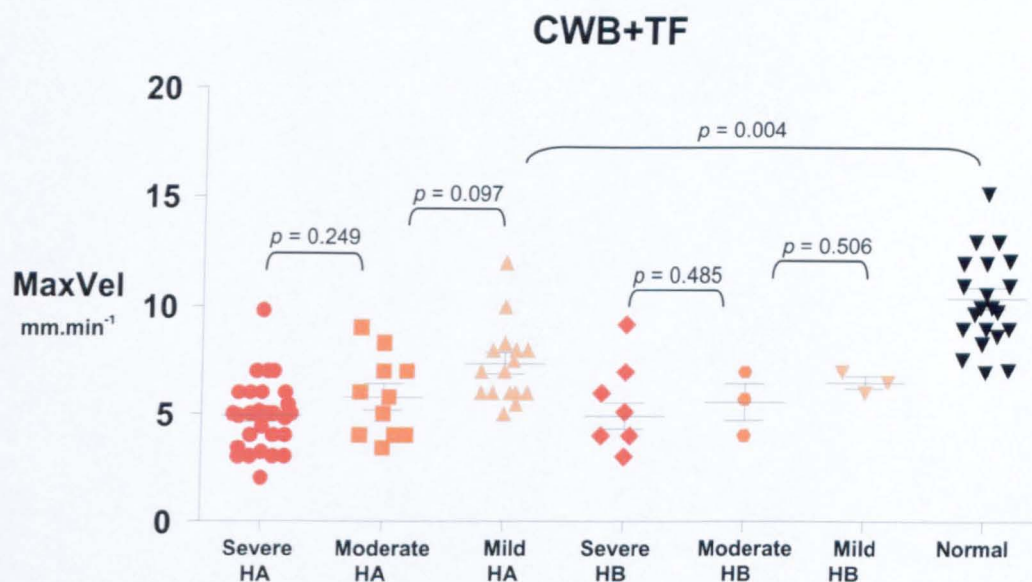


Figure 3.11 ROTEM® MaxVel comparison in CWB+TF

The graph depicts MaxVel parameter in severe, moderate, mild HA and HB patients and the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

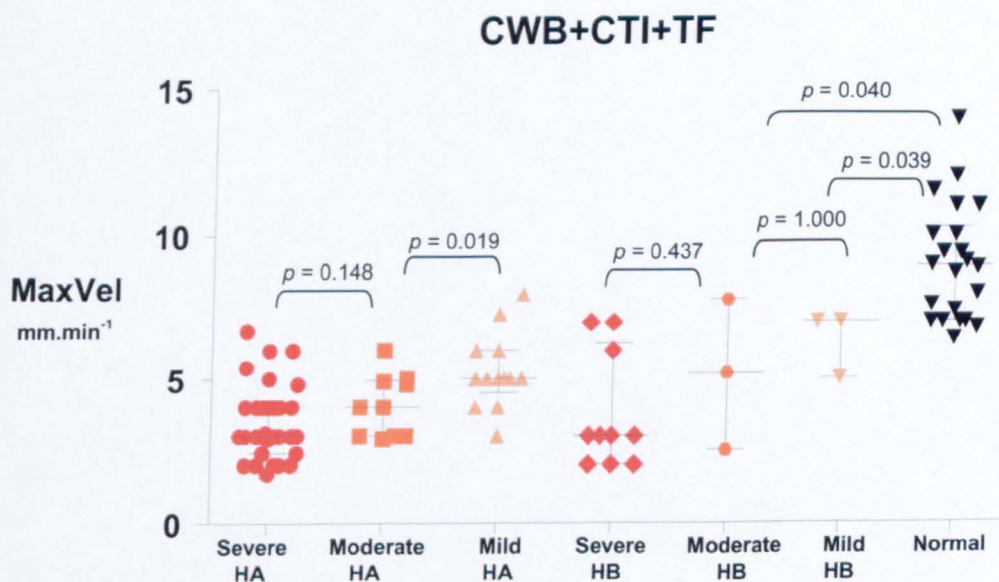


Figure 3.12 ROTEM® MaxVel comparison in CWB+TF+CTI

The graph depicts MaxVel parameter in severe, moderate, mild HA and HB patients and the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

3.6.2.2. Time to maximum velocity

The tMaxVel parameter was analysed and compared to the control group (Table 3.2). tMaxVel was significantly prolonged in all HA and HB groups compared to the control group (the highest $p = 0.048$) (Figures 3.13, 3.14, and 3.15). To determine any difference between the severe and moderate HA groups in the different test categories, the Mann-Whitney U test was performed and a significant difference was found between the severe and the moderate HA in CWB test category only ($p = 0.002$). The comparison between the moderate and mild HA patient group showed a significant difference in the CWB and CWB+TF test categories ($p = 0.010$ and 0.007 respectively) but not in CWB+CTI+TF test category ($p = 0.538$). In HB groups, no significant difference was found between severe and moderate or moderate and mild HB groups. This could be explained by small number of participants in these groups and also the fact that the median FIX activity levels in these group were very close to each other (median FIX = 2.0 IU/dL in moderate HB, and 5.0 IU/dL in mild HB).

Wilcoxon rank test compared the difference between test categories, and showed significant difference in tMaxVel of severe HA in all test categories. In moderate HA, the difference between tMaxVel in CWB and CWB+TF test categories was significant ($p = 0.008$), but not between CWB and CWB+CTI+TF test categories ($p = 0.109$). There was also significant difference between tMaxVel of CWB+CTI+TF and CWB+TF test categories ($p = 0.007$).

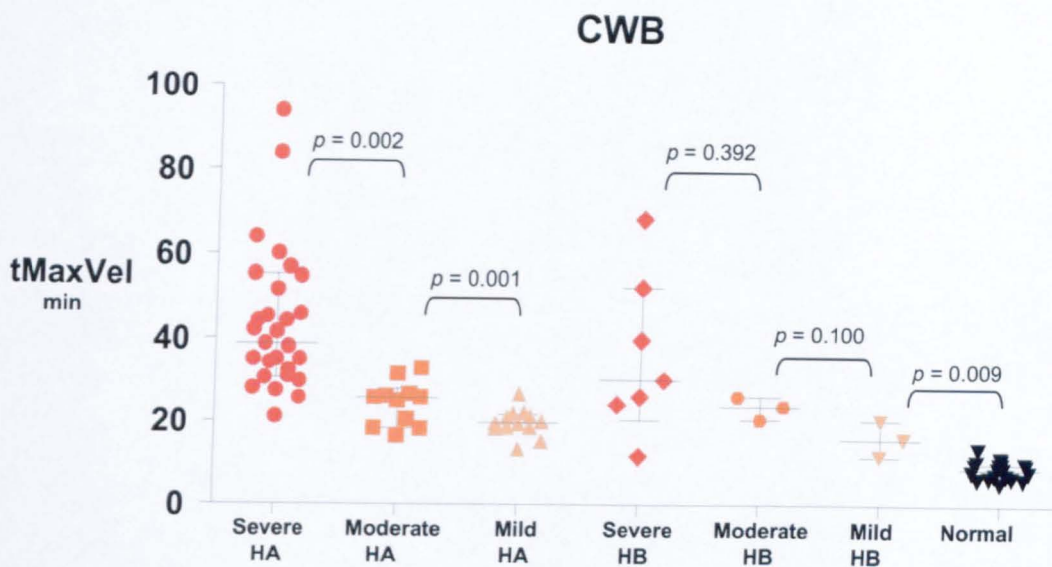


Figure 3.13 ROTEM® tMaxVel parameter in CWB

tMaxVel in severe, moderate, mild HA and HB compared to the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

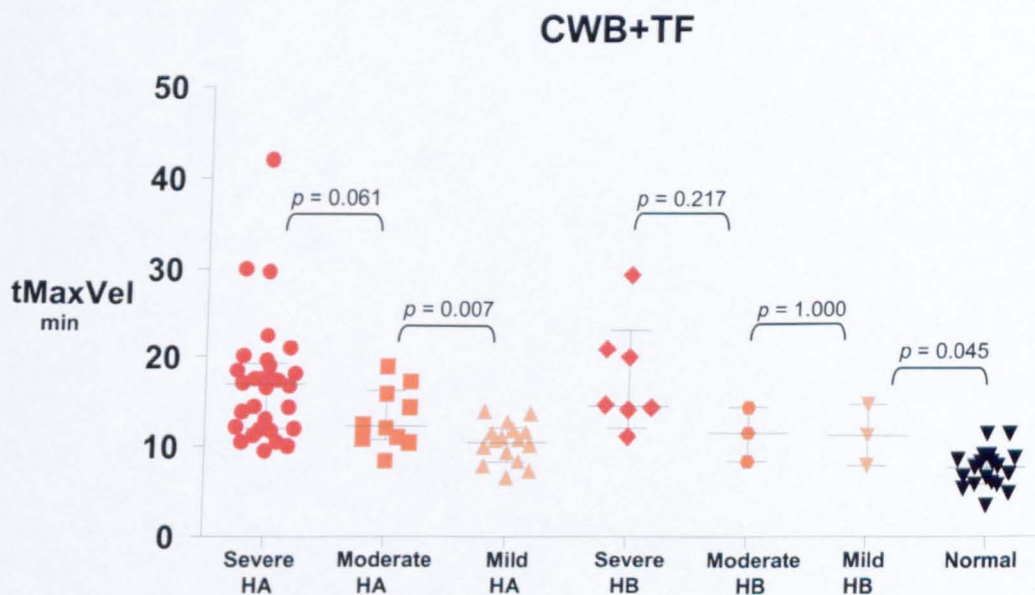


Figure 3.14 ROTEM® tMaxVel parameter in CWB+TF

tMaxVel in severe, moderate, mild HA and HB compared to the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

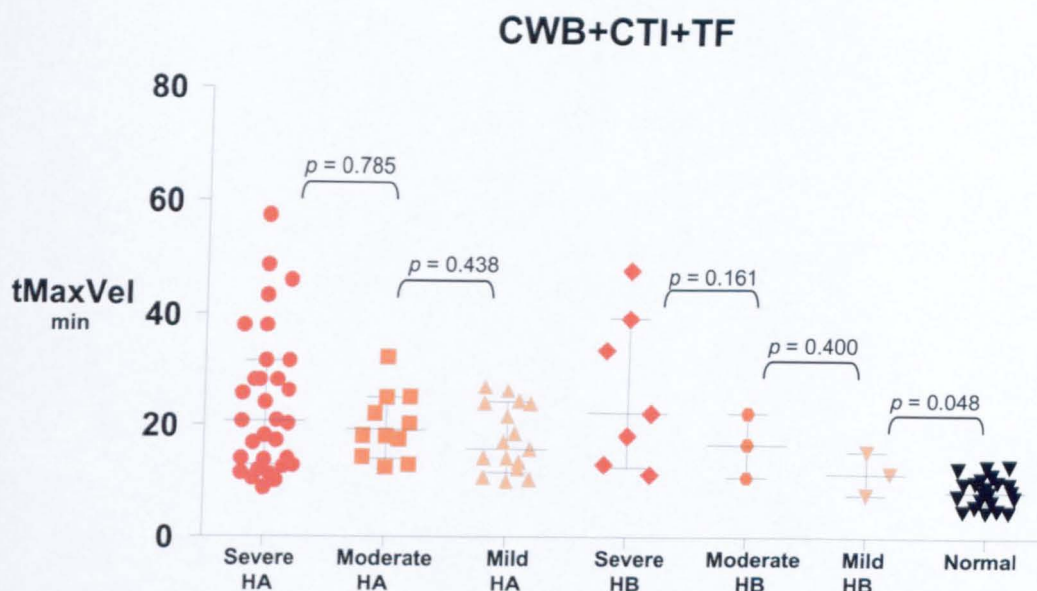


Figure 3.15 ROTEM® tMaxVel parameter in CWB+TF+CTI

tMaxVel in severe, moderate, mild HA and HB compared to the control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

3.6.2.3. Area under the curve

The AUC of ROTEM® velocity curve was measured and the median and 5th- 95th percentiles were calculated (Table 3.2). The Mann-Whitney U test showed significant difference between control group and HA or HB groups in CWB (the highest p =0.023) and CWB+TF (the highest p =0.028), but not in CWB+CTI+TF (the lowest p =0.079) test category. There was no significant difference between severe, moderate, mild HA or HB groups in any test category (Figures 3.16, 3.17, and 3.18). The results showed that AUC parameter was not sensitive enough to distinguish between control group and any of patient groups in any test category.

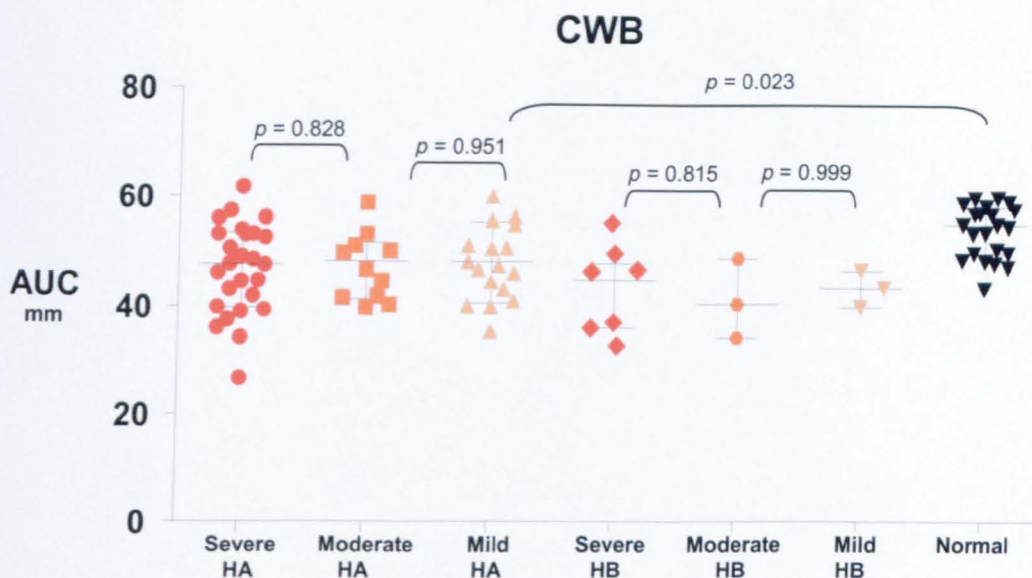


Figure 3.16 ROTEM® AUC in CWB

AUC parameter in severe, moderate, mild HA and HB compared to the control group in CWB test category. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

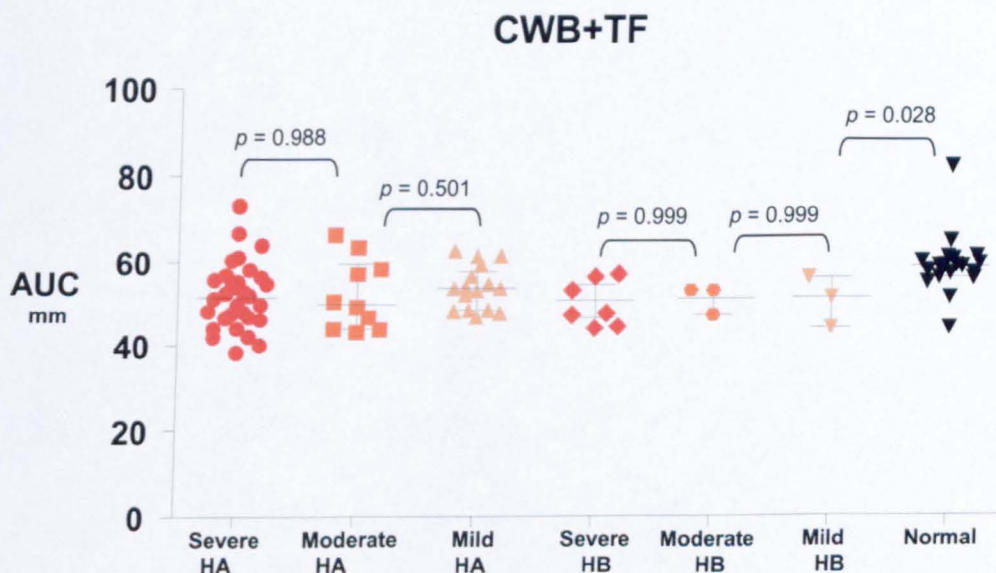


Figure 3.17 ROTEM® AUC in CWB+TF

AUC parameter in severe, moderate, mild HA and HB compared to the control group in CWB+ TF test category. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

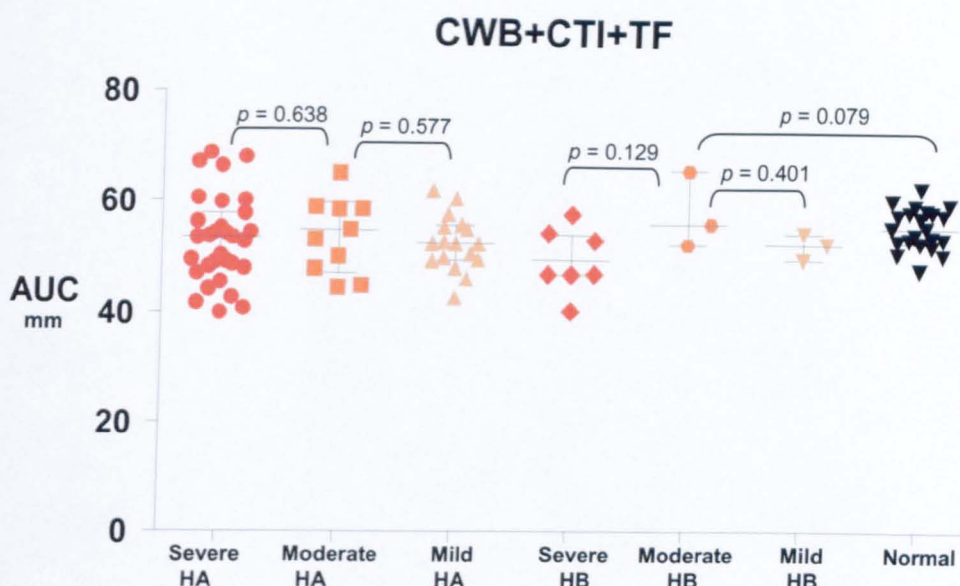


Figure 3.18 ROTEM® AUC in CWB+TF+CTI

AUC parameter in severe, moderate, mild HA and HB compared to the control group in CWB+CTI+TF test category. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

The variability within groups showed a high degree of heterogeneity, particularly in severe HA and HB. This was more pronounced in CWB+TF test category in which the standard deviation ($SD = 1.6$) was higher than CWB and CWB+CTI+TF test categories ($SD = 1.0$ and 1.3 respectively). However, four severe, four moderate and eight mild HA were within the reference range in CWB+TF test category as were one severe, one moderate, and two mild HA in CWB, and one severe, no moderate and two mild HA in CWB+CTI+TF test category.

3.6.2.4. Correlation of ROTEM® parameters with FVIII levels

The Spearman correlation coefficient was performed between ROTEM® parameters and the corresponding FVIII level of mild and moderate HA (Appendix 1) (median = 10 IU/dL , 5th- 95th percentile range $1.4\text{-}39.6 \text{ IU/dL}$) showed that the MaxVel

correlated significantly with the level of FVIII in CWB+CTI+TF ($r = 0.805$, $p < 0.0001$), and CWB ($r = 0.610$, $p = 0.004$) test categories only. The linear regression analysis showed a significant coefficient of determination ($r^2 = 0.648$, $p < 0.0001$) (Figure 3.19). Correlation of the tMaxVel with the FVIII level was only found in CWB test category ($r = -0.625$, $p = 0.003$) and to a lesser degree in CWB+TF ($r = -0.487$, $p = 0.025$). This correlation was performed on combined moderate and mild HA groups to increase the number of individuals and to produce a statistically valid outcome.

The correlation between tMaxVel and APTT (Appendix 1) was also significant ($r = 0.726$, $p < 0.0001$). Spearman correlation between FVIII levels of mild and moderate HA and APTT were less significant than the MaxVel in CWB+CTI+TF ($r = -0.710$, $p < 0.0001$). The linear regression analysis showed a significant coefficient of determination ($r^2 = 0.648$, $p < 0.0001$) (Figure 3.20). There was no correlation between one-stage FIX clotting assay and any of the ROTEM[®] parameters.

Linear regression curve FVIII vs MaxVel

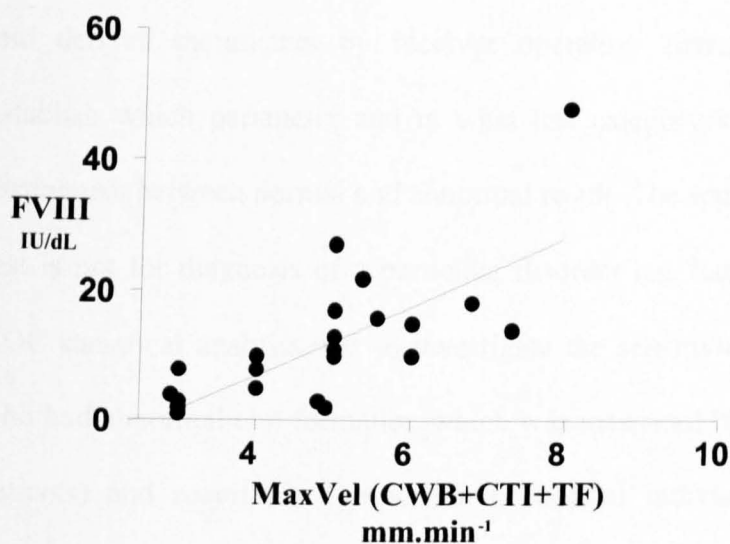


Figure 3.19 Linear regression FVIII vs MaxVel in CWB+CTI+TF

Linear regression analysis was performed to compare FVIII activity levels and MaxVel in moderate and mild HA ($n = 27$) in CWB+CTI+TF test category ($r^2 = 0.648$, $p < 0.0001$).

Linear regression curve APTT vs MaxVel

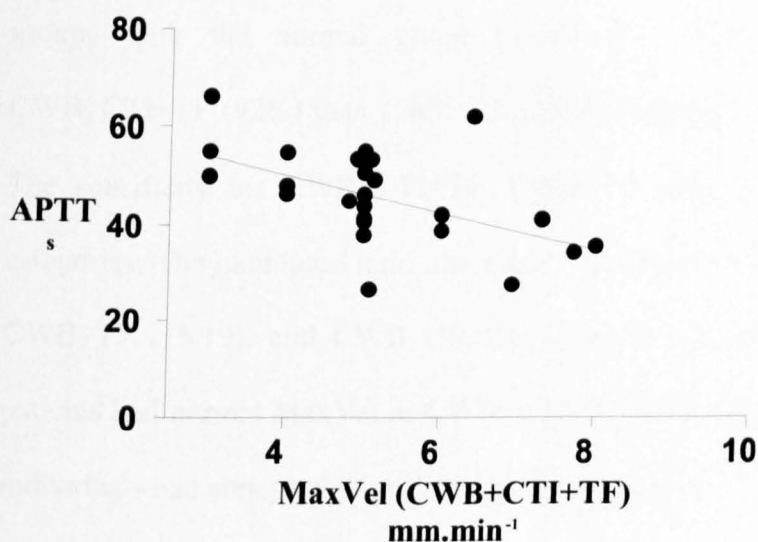


Figure 3.20 Linear regression APTT vs MaxVel in CWB+CTI+TF

Linear regression analysis was performed to compare APTT and MaxVel in moderate and mild HA ($n = 27$) in CWB+CTI+TF test category ($r^2 = 0.504$, $p < 0.0001$).

3.6.2.5. The sensitivity and specificity of ROTEM[®] parameters

The sensitivity, specificity and likelihood ratio were calculated for the conventional and derived parameters by receiver operating characteristic (ROC) analysis to establish which parameter and in what test category was sensitive and specific to distinguish between normal and abnormal result. The sensitivity and specificity in this test is not for diagnosis of a particular disorder e.g. haemophilia. The aim of using ROC statistical analysis was to investigate the sensitivity (proportion of individuals who had abnormal clot formation which was measured by ROTEM[®] i.e. haemophilia patients) and specificity (proportion of normal individuals who had normal clot formation which was measured by ROTEM[®]) of ROTEM[®] parameters as a screening test for hypocoagulable states, and to establish whether the use of CTI would improve the sensitivity of the test. The ROC analysis was performed on all HA and HB patients ($n = 70$) in each test category and compared to 22 normal individuals.

The ROC was calculated for MaxVel in all three test categories, comparing the patient groups with the normal group (Table 3.3). The sensitivity was higher in CWB+CTI+TF (92%) than CWB+TF (85%), and slightly higher than CWB (90.41). The specificity for CWB+CTI+TF, CWB+TF, and CWB were 95% in all three categories. The likelihood ratio, therefore, was higher in CWB+CTI+TF (20.31), than CWB+TF (16.19), and CWB (18.08). These results indicate that only 8% of 70 patients had normal MaxVel in CWB+CTI+TF test category, and only 5% of normal individuals had abnormal MaxVel in the same category.

Table 3.3 ROTEM[®] MaxVel sensitivity and specificity

Test Category	Sensitivity %	Specificity %	Likelihood ratio
CWB+CTI+TF	92	95	20.31
CWB+TF	85	95	16.19
CWB	90	95	18.08

The sensitivity, specificity and likelihood ratio of MaxVel in all HA and HB ($n = 70$) in three categories was calculated by comparing with normal individuals ($n = 22$). CWB = citrated whole blood, CTI = corn trypsin inhibitor, TF = tissue factor

To avoid skewed results, the ROC analysis repeated for mild HA ($n = 16$) only, by comparing to the normal individual group ($n = 22$) in all three test categories and for all parameters (Table 3.4). The results showed CFT in CWB+CTI+TF was the most sensitive conventional parameter for distinguishing normal from abnormal across the three test categories with the sensitivity of 85%, the specificity of 95% and likelihood ratio of 18.62. The sensitivity, the specificity of the α° was similar to the CFT (85%) in CBW+CTI+TF but less specific (91%) than the CFT (95%). The sensitivity and likelihood ratio of MaxVel parameter in CWB+CTI+TF category was higher in comparison to the other test categories. The sensitivity, the specificity, and likelihood ratios in HB group were 81%, 95%, and 17.88 respectively.

The tMaxVel was the most sensitive parameter of ROTEM[®] in mild HA CWB category with sensitivity of 100% and specificity of 95%. In the HB group, the ROC showed similar results with HA with 100% sensitivity, 95% specificity and 20.0 likelihood ratio. In contrast, AUC was the least sensitive and specific parameter among ROTEM[®] parameters in all test categories.

Table3.4. Sensitivity and specificity of ROTEM® MaxVel

ROTEM® Parameters	Category	ROC (Mild HA patients)		
		Sensitivity %	Specificity %	Likelihood Ratio
CT s	CWB	62	95	12.31
	CWB+ TF	67	95	12.67
	CWB+CTI+TF	62	95	13.54
CFT s	CWB	69	90	6.92
	CWB+ TF	60	95	11.40
	CWB+CTI+TF	85	95	18.62
α°	CWB	46	95	9.23
	CWB+ TF	53	95	10.13
	CWB+CTI+TF	85	91	9.31
MaxVel mm.min ⁻¹	CWB	77	95	15.38
	CWB+ TF	57	95	10.86
	CWB+CTI+TF	85	95	18.62
tMaxVel min	CWB	100	95	20.0
	CWB+ TF	71	89	6.79
	CWB+CTI+TF	54	95	11.85
AUC mm	CWB	38	95	8.46
	CWB+ TF	74	79	3.44
	CWB+CTI+TF	46	95	9.23

The ROC was analysed in all three categories for all parameters of ROTEM® in mild HA haemophilia patients (*n* = 16) and normal group (*n* = 22). CT = clotting time, CFT = clot formation time, MaxVel = maximum velocity, tMaxVel = time to maximum velocity, AUC = area under the first derivative curve, CWB = citrated whole blood, CTI = corn trypsin inhibitor, TF = tissue factor

3.7. Discussion

The ROTEM[®] whole blood analyser was used in this study to examine changes in viscoelastic properties of whole blood clot formation in haemophilia patients. Thrombelastography/thromboelastometry has been used for several decades (Luddington *et al.*, 2005). In recent years, however, they have become a more useful tool for the assessment of whole blood haemostasis in laboratories. ROTEM[®] is not routinely used to monitor patients with haemophilia as the ROTEM[®] tracing in this group of patients takes a long time to produce a result, and not all the parameters can be extracted as a result of the prolonged clotting time. However, the use of a small amount of TF for initiation of coagulation has made this technique useful as it more clearly mimics the *in vivo* situation, particularly in haemophilia patients with inhibitors (Ingerslev *et al.*, 2003; Sørensen & Ingerslev, 2004b).

The aim of this study in this chapter was to investigate the effect of leukocytes, red blood cells and platelets, on the changes to the viscoelastic properties of clot formation in patients with haemophilia, and to establish the most sensitive and specific parameters to distinguish between normal and abnormal results. Once the most sensitive and specific parameter was identified, its correlation with the clinical phenotype was analysed (see Chapter 6).

Although other groups have performed similar studies, the number of participants in those studies was less than our study i.e. Lewis *et al.* (2007) investigated 12 severe HA, and ven Veen *et al.* (2009) assessed 29 HA patients.

Furthermore, the effect of preanalytical variables, especially, the effect of CTI on whole blood clotting assay were also not thoroughly investigated. Contact activation during sampling and sample processing can have a significant influence on the results. The results showed significant differences between MaxVel in CWB+TF and CWB+CTI+TF, and also between tMaxVel in CWB and CWB+CTI+TF test categories. This may indicate that the influence of contact activation, in addition to the effect of TF, may cause overestimation of results in MaxVel and shortening the tMaxVel in CWB+TF.

Consistent with other studies, this study showed that severe HA and HB patients had a very prolonged CT and CFT when compared to a control group under all test conditions. The MaxVel was markedly depressed in severe haemophilia but increased proportionally in patients with moderate and mild haemophilia. This was shown by a good correlation between FVIII and MaxVel in moderate and mild HA. TF initiation of the reaction improved the tracing record but variability increased dramatically, particularly in the severe HA group. This may, in part, be related to the overall contribution of other components of blood, including red cells, platelets and white cells to clot formation or the ability of ROTEM[®] to identify the influence of the changes in factor VIII levels at < 1.0 IU/dL on the coagulation system.

In the CWB category, CT, α° , and tMaxVel differentiated between severe and moderate HA in ($p = 0.0019, 0.0401$, and 0.002 respectively). However, there were number of crossover results which the ROTEM[®] results were not in agreement with the current classification of severe, moderate, and mild haemophilia based on the coagulation factor level activity. This shows that the global assays i.e.

thromboelastometry, may be influenced by other components of blood and may not strongly correlate with the current definition of severity of haemophilia.

The tMaxVel could also differentiate between the moderate and mild HA ($p = 0.0101$). The MaxVel parameter could differentiate between moderate and mild HA ($p = 0.0082$). However, the sensitivity and specificity of the tMaxVel parameter was the highest amongst all six parameters in all categories (100% sensitivity and 95% specificity). The tMaxVel correlated with the FVIII level in CWB test category ($r = -0.625$, $p = 0.003$) and the data suggest that tMaxVel in the CWB category is the most useful parameter of ROTEM[®] for predicting the severity of haemophilia. The significant correlation between APTT and tMaxVel ($r = 0.726$ $p < 0.0001$) suggests that this parameter may reflect the contact activation in the CWB category therefore may not truly represent *in vivo* haemostasis.

In CWB+TF category, only tMaxVel differentiated between the moderate and mild HA ($p = 0.0101$). The correlation between the FVIII level and the ROTEM[®] parameters in this category was not significant. The sensitivity and specificity of the various parameters in this test category were also inferior to the parameters of other test categories, as discussed in section 3.6.2.5.

In CWB+CTI+TF category, CFT differentiated between severe and moderate HA ($p = 0.0211$), and was the most sensitive and specific parameter amongst the three conventional parameters. The MaxVel parameter could differentiate between moderate and mild HA in this category ($p = 0.0192$). The sensitivity and specificity of the MaxVel were also high (85% sensitivity, 95% specificity) with a significant

correlation to the FVIII levels ($r = 0.805, p < 0.0001$). The MaxVel seems to be the most useful parameter of ROTEM[®] in the CWB+CTI+TF category in predicting the severity of haemophilia.

This study was the first (at the beginning of the study) to conduct ROTEM[®] analysis on haemophilia patients, using CTI and TF to closely mimic *in vivo* haemostasis in whole blood. One of the major variations in the performance and interpretation of ROTEM[®] measurement is the contribution of preanalytical variables. These variables include techniques used for specimen collection, transport, and storage, all which may introduce changes due to activation of the coagulation system and not related to the underlying disorder in the ROTEM[®] traces, affecting the precision and accuracy of the test. Coagulation activation of blood by the contact pathway can be abolished by inhibiting contact activation through the use of CTI. Therefore, the coagulation initiation will only be dependent on the trigger used, which in this instance was a minute amount of TF added to the ROTEM[®] cup. The use of CTI in ROTEM[®] improved the sensitivity of parameters.

3.8. Conclusion

In conclusion, ROTEM[®] analysis of citrated whole blood under two test categories of CWB and CWB+CTI+TF was most useful for identifying the heterogeneity of the patient's global coagulation profile. The MaxVel in CWB+CTI+TF test category differentiated the severity of haemophilia between populations, i.e. severe, moderate, and mild; and had strong correlation with the FVIII level of individuals. Where TF is used to trigger coagulation, in order to mimic *in vivo* clot formation, this study

showed that the use of CTI is essential to improve the sensitivity and specificity of the test.

The tMaxVel in CWB category can also reliably distinguish individuals with HA with 100% sensitivity from normal population, however, its advantage over conventional assays such as APTT remains controversial as the initiation of clot formation, similar to APTT, is through the contact pathway.

Although the results showed a significant difference between severe, moderate and mild groups, the number of crossovers cannot be ignored. This may prove that not all severe, moderate, or mild haemophilia patients have the same coagulation potential and modifying elements may play a role in altering the phenotypic expression.

Therefore, a modified ROTEM[®] analysis using CTI and TF, mimicking *in vivo* haemostasis may give additional information over conventional coagulation assays and contribute to the management of haemophilia patients including the prediction of the clinical phenotype.

Chapter 4 **Thrombin generation test**

4.1. Introduction

Thrombin is one of the central enzymes in blood coagulation and both the rate of thrombin formation and the total amount of thrombin formed can be considered to be a reflection of the potential coagulation activity in plasma (Hemker *et al.*, 2002). Activation of the coagulation cascade *in vivo* is thought to occur through complex formation of circulating FVIIa and vessel wall exposed TF, whereas a second pathway, the so-called “contact activation” route, can be stimulated *in vitro* (Biggs *et al.*, 1953). The classical laboratory methods to determine coagulation defects in both pathways, *e.g.* the PT for the TF/FVIIa route and the APTT for contact activation, are limited in their ability to simulate the dynamics of clot formation because the tests measure only the initial thrombin formation required for fibrin formation (95% thrombin is generated after initial clot formation) (Mann *et al.*, 2003a). Therefore, the coagulability of blood is better quantified by its thrombin generation capacity than by measuring the clotting times (Aledort, 2003).

The TGT was first introduced by Macfarlane and Biggs in 1953 and its use in individuals with haemophilia was one of the first clinical applications of the assay. In their experiment, in brief, prothrombin was removed from blood samples with $\text{Al}(\text{OH}_3)$, then from the prothrombin-free samples, fibrinogen was separated into another sample collection tube to make a fibrinogen solution. Blood samples for testing were then sub-sampled into the fibrinogen solution and the time taken for fibrinogen to clot was measured. The concentration of thrombin in the whole blood at each time of sampling was then obtained from the thrombin-dilution curve.

Hemker *et al.* introduced substantial modifications, which made the test easier and more precise to perform (Hemker *et al.*, 2003). The quantity of thrombin generated is plotted against time to obtain a curve from which four parameters can be determined: 1) area under the curve (AUC) or endogenous thrombin potential (ETP); 2) lag time, which represents the initiation of coagulation by generation of a small amount of thrombin (< 5%) (Hemker & Beguin, 1995); 3) Peak thrombin generation (peak height), represent the maximum amount of thrombin generated; 4) time to peak height of thrombin generation.

4.2. Aims

In haemophilia patients, a correlation is usually observed between the clinical expression of the disease and the plasma FVIII/FIX activity. However, there are some patients with haemophilia where the bleeding tendency is not proportional to their plasma FVIII/FIX concentration. It is known from clinical experience that approximately 10-15% of severe haemophilia patients, despite FVIII/FIX plasma levels < 1.0 IU/dL, can exhibit a milder bleeding phenotype and do not have haemophilic arthropathy (Bolton-Maggs & Pasi, 2003). From a clinical perspective, it would be extremely useful to have a laboratory test that can accurately predict an individual's coagulation potential and thus their bleeding tendency. However, there is still no validated laboratory test available to predict the overall coagulation status of an individual. The aim of the study in this chapter was to critically evaluate the use of thrombin generation in evaluation of overall coagulation potential in haemophilia.

This chapter describes TGT performed on haemophilia A and B individuals, and compares the assay in four different test categories:

- PPP: platelet poor plasma to analyse coagulation factors.
- PRP: platelet rich plasma, in which the platelet count was adjusted by PPP to give a platelet count of $150 \times 10^9 /L$, to analyse the effect of platelets on thrombin generation.
- PPP+CTI: where samples were collected into tubes with added CTI to eliminate the contact activation contribution to thrombin generation.
- PRP+CTI.

For each test category statistical analysis was performed using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Mann-Whitney *U* test was performed to compare parameters within and between patients group and the control group. Wilcoxon rank test was performed to compare paired parameters in the three test categories for each patient group, and Kruskal-Wallis nonparametric ANOVA test was performed to analyse the variance between three test categories. The correlation coefficient was determined using the Spearman rank method. The ROC was performed to evaluate the sensitivity and specificity of the TGT. In all statistical analysis, a *p* value of < 0.05 was considered significant (All severe haemophilia samples included in the statistical analysis had FVIII/FIX activity level of < 1.0 IU/dL, as measured by the one stage clotting assay).

TGT in-house assay was also compared to the Calibrated Automated Thrombogram[®] (CAT) assay (section 4.4.2, Table 4.4) to investigate the correlation of in-house assay and one of the commercially available methods. The comparison was made only in the PPP+CTI test category, as the CAT was not available in our laboratory at the

beginning of this study, therefore, PRP samples which had to be tested as soon as prepared could not be tested by the CAT method.

4.3. Study subjects

Ninety seven patients were consented for the study, and in-house TGT data for further analysis was available for 73 patients. This included 36 severe HA, eight moderate HA, 15 mild HA, seven severe HB, three moderate HB, and four mild haemophilia B. The age range was between 18 and 90 years (mean 43, median 41 years). The control group comprised 22 healthy male individuals aged between 22 and 62 (mean 40, median 39 years). All severe haemophilia samples included in the statistical analysis had FVIII or FIX activity level of < 1.0 IU/dL, as measured by the one stage clotting assay. The study was approved by the ethics committee of the Royal Free Hospital, and informed consent was obtained from patients and healthy individuals.

4.4. Results

The in-house TGT results were expressed by relative fluorescence unit (RFU). The RFU was calculated as a percentage of the normal value to enable comparison within and between different TG assays. In-house TGT parameters comprised peak height (PH) (the maximum amount of thrombin generated), the area under the curve (AUC), and the time to the peak height (TP) for the controls and the haemophilia patients. The lag time could not be measured in in-house method (the in-house TGT parameters, by definition, are same as the parameters which were described by Hemker *et al.*, however, to distinguish between the in-house and CAT method, different abbreviation was used). The results showed a nonparametric distribution for the majority of the

parameters, therefore median and the range (based on 5th- 95th percentiles) was calculated for each parameter (Table 4.1).

Table 4.1 Thrombin generation test (in-house) parameters

TGT	Test Category	Normal <i>n</i> = 22 Median (5 th -95 th percentile)	Severe HA <i>n</i> = 36 Median (5 th -95 th percentile)	Moderate HA <i>n</i> = 8 Median (5 th -95 th percentile)	Mild HA <i>n</i> = 15 Median (5 th -95 th percentile)	Severe HB <i>n</i> = 7 Median (5 th -95 th percentile)	Moderate HB <i>n</i> = 3* Median (5 th -95 th percentile)	Mild HB <i>n</i> = 4* Median (5 th -95 th percentile)
PH %	PPP+CTI	95 (66-152)	16 (6.4-31.5)	28.5 (16-50)	32.3 (17.8-60.5)	23 (14-31)	27 (12-37)	26.5 (19-50)
	PPP	97 (64-159)	19.6 (6.7-39.5)	44.4 (18.2-47.9)	42.1 (16.6-84.1)	13.8 (11.9-29.4)	14.4 (13.9-29.1)	37.8 (31.2-50.5)
	PRP+CTI	97 (67-133)	28.8 (9.3-57.6)	30.8 (15.2-70)	41.6 (29.4-65.2)	34 (22.9-34.5)	41.4 (30.8-51.9)	55.2 (44-66.7)
	PRP	104 (65-134)	31.7 (9.5-56.9)	36.3 (24.9-65.4)	53.5 (33.1-69.7)	26 (19-30)	26 (24.5-27.5)	37 (35.6-38.3)
AUC %	PPP+CTI	90.5 (78-133)	34.4 (12.1-56.7)	40 (32-70)	53.3 (27.1-90.8)	41 (33-69)	43 (36-53)	38 (29-48)
	PPP	100 (72-124)	38.8 (14.6-61.5)	66.5 (66.1-66.8)	55.5 (27.9-104.3)	36.7 (27.1-68.1)	31.4 (26.2-36.6)	44.3 (34.9-50.7)
	PRP+CTI	103 (71-115)	37.2 (10.0-71.4)	39.7 (21.3-64.4)	49.4 (28.6-83.8)	31.6 (26.8-45.4)	40.7 (15.1-43)	50 (19.9-70.3)
	PRP	93 (86-114)	39.7 (9.0-77.3)	54.7 (35.0-89.2)	76.9 (52.0-93.7)	34 (22.9-34.5)	46.3 (35-57.5)	55.2 (44-66.4)
TP min	PPP+CTI	12 (9-15)	20.5 (14.4-52.6)	18.3 (11.5-28)	17.5 (13-22)	24 (15.5-27)	18.5 (15-37)	14.3 (13.5-15)
	PPP	12 (9-15.5)	20 (13.5-54.5)	12.8 (11.5-14)	15 (13-20)	22 (15-25)	22 (18.5-25.5)	13.5 (12.5-15)
	PRP+CTI	29 (20.5-49)	50.8 (37-61)	47.5 (23.5-58)	38.5 (32.5-56)	59 (55-59)	36.5 (22.5-58)	35 (32.5-37.5)
	PRP	22 (15-41)	50 (34.5-60)	41 (25-47.5)	32.5 (24.5-41)	58 (58-60)	46.3 (35-57.5)	34.3 (32.5-36)

The median and the range (5th- 95th percentiles) for in-house TGT were calculated in control group and Haemophilia patients in four categories. TGT = thrombin generation, PH = peak height, AUC = area under the thrombin generation curve, TP = time to peak thrombin generation, PPP = platelet poor plasma, PRP = platelet rich plasma, CTI = corn trypsin inhibitor, HA = haemophilia A, HB = haemophilia B.
 *where the number of samples was not statistically significant, minimum and maximum values were reported

The reference range was established using a control group comprised of 22 male healthy volunteers. Within and between assay precision were performed by using two normal individuals who had the nearest thrombin generation values to the mean of the reference range. Intra- and inter-assay coefficient of variation was calculated by performing 10 duplicate assays on each sample in one run and 10 duplicate assays on the samples from the same normal individual on different days during a period of 6 months to take into account the possibility of fluctuations of coagulation potential in

the same donor. The mean of the SD of 10 replicates RFU results was divided by the mean of 10 replicates. The normal individual with the lowest CV (AUC = 11%, PH = 7%, TP = 12%) then kindly volunteered to donate blood samples throughout the study. This sample was used in each assay as a control and to normalise the raw data in each run.

4.4.1. In-house thrombin generation test (TGT)

4.4.1.1. Peak height

The median and the range (5th - 95th percentiles) of peak height (PH) were calculated for the patient groups and normal controls in each test category (Table 4.1). PH in the patients in each test category when compared to the control group, showed a significant decrease in all patient groups.

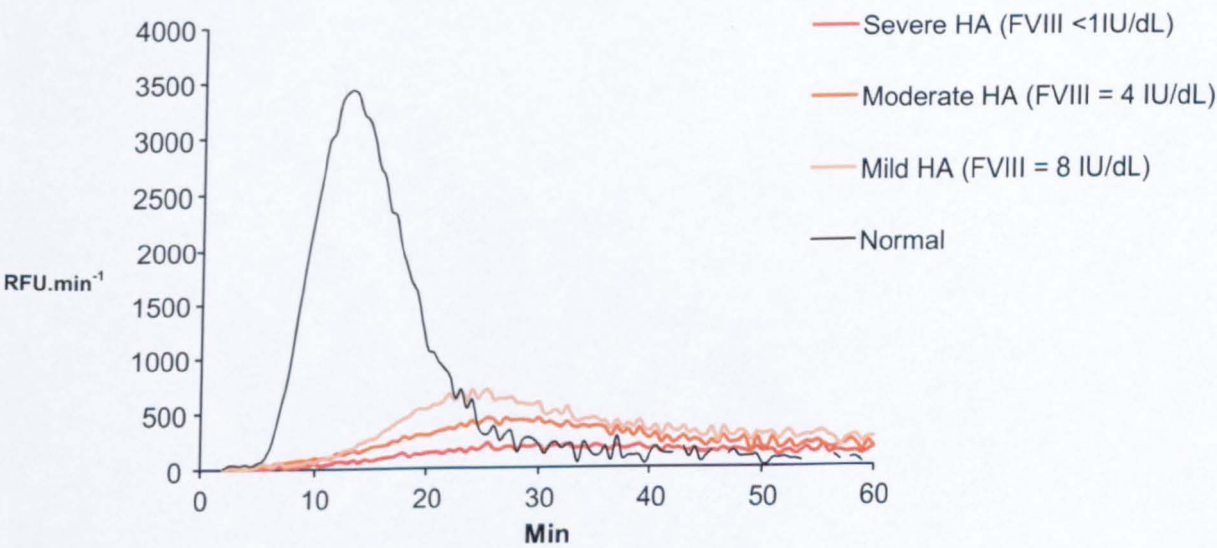


Figure 4.1 Thrombin generation curve

Peak Height in-house thrombin generation curve showing a typical relative fluorescence unit (RFU/min) over time (Min) in severe, moderate, mild haemophilia A, and normal samples.

Using the Mann-Whitney U statistical test a significant difference ($p < 0.0001$) was found between of the control and patient groups in the 4 categories (Figures 4.2, 4.3, 4.4, and 4.5).

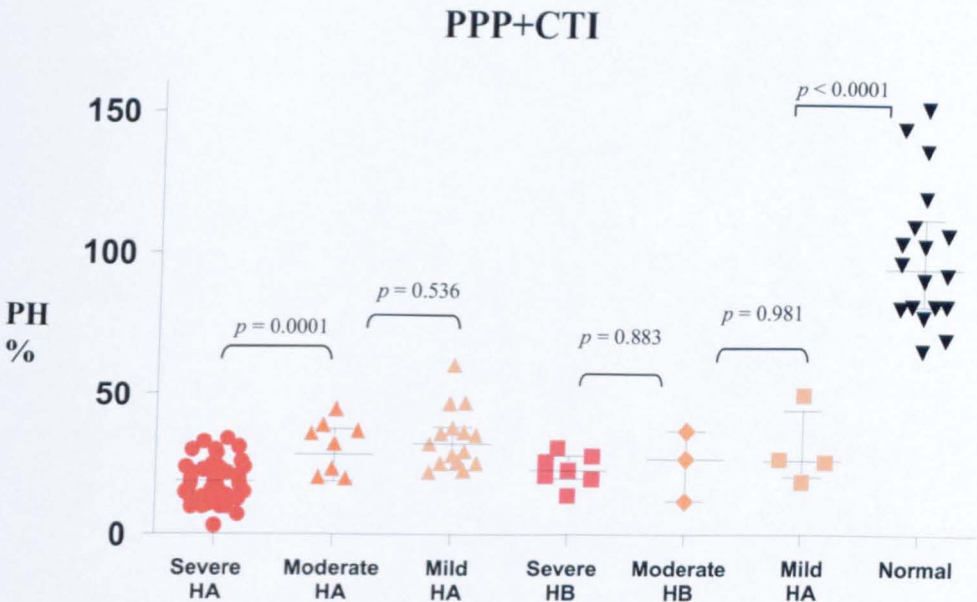


Figure 4.2 Peak height thrombin generation (PPP+CTI)

Peak height TGT PPP+CTI (expressed as a percentage of the control sample) in severe, moderate and mild HA and HB compared to normal group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

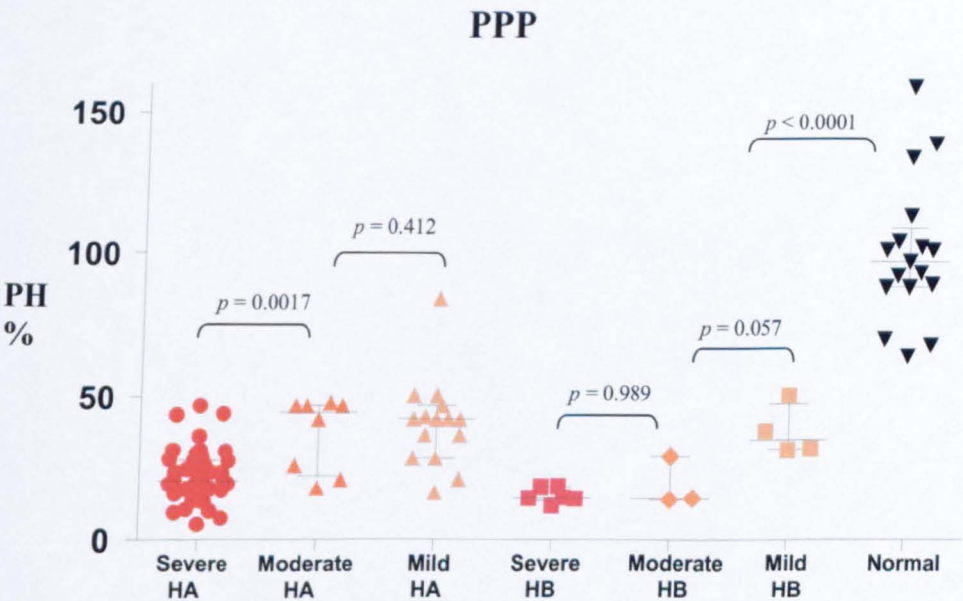


Figure 4.3 Peak height thrombin generation (PPP)

Peak height TGT PPP (expressed as a percentage of the control sample) in severe, moderate and mild HA compared to normal group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

Mann Whitney statistical analysis was performed to compare median difference of PH between mild, moderate, and severe HA. Significant differences were found between PH of severe and moderate HA patients in PPP and PPP+CTI test categories. ($p = 0.0017$ and 0.0001 respectively). However, no significant difference was found between PH of mild and moderate groups ($p = 0.412$ and 0.536 respectively). The median FVIII activity levels, which were measured by one-stage clotting factor assay (Appendix 1), were 3.0 IU/dL (5th- 95th percentile 1-5.0 IU/dL) and 13.0 IU/dL (5th- 95th percentile 8-48.0 IU/dL) for moderate and mild HA respectively. These results showed that the mild HA group have negative skewed results (skewed toward left), which may partly explain the lack of significant difference between the two groups. On the other hand, this may show insensitivity of the TGT in differentiating between the two groups. However, the fundamental question remains as to whether the TGT should be correlated to the clinical phenotype or to a single coagulation factor activity, if it is to be of diagnostic value. The aim of employing such global assays i.e. ROTEM[®] or TGT, is to take into account other modifiers of coagulation potential, and to prove or disprove whether such an effect exists. Perhaps one should expect a lesser degree of correlation with measured FVIII/FIX, or a new classification of severity based on global assays, if such tests proved useful. In PRP and PRP+CTI categories, no significant differences were found between severe and moderate, or mild and moderate groups (Figures 4.4, and 4.5).

Using Wilcoxon paired test, no significant difference was found between the PH parameter of PPP and PPP+CTI ($p = 0.139$), and PRP and PRP+CTI ($p = 0.726$) test categories (paired data on all patient groups). However, a significant difference was

found between the PH parameter of PPP and PRP ($p = 0.0004$), and PPP+CTI and PRP+CTI ($p = 0.0221$) test categories.

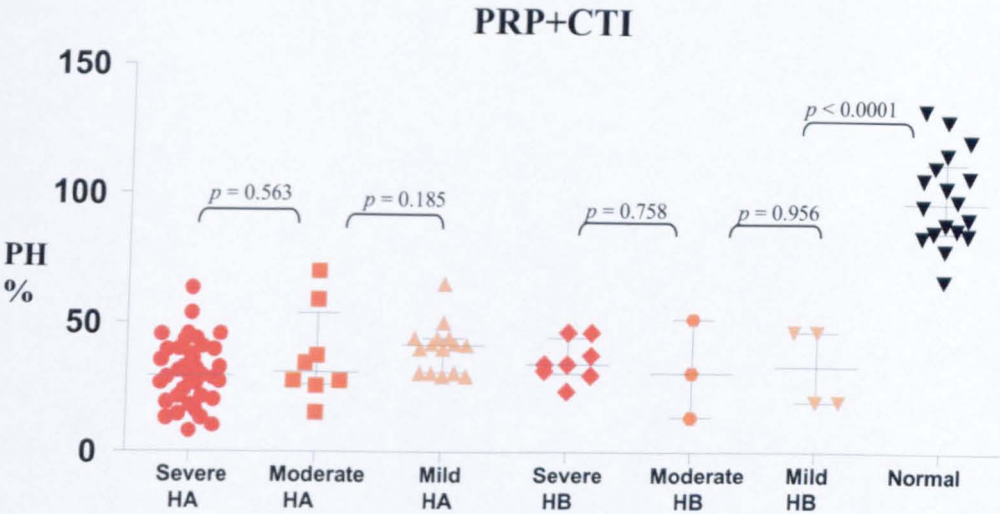


Figure 4.4 Peak height thrombin generation (PRP+CTI)
Peak height TGT PRP+CTI (expressed as a percentage of the control sample) in severe, moderate and mild HA compared to normal group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

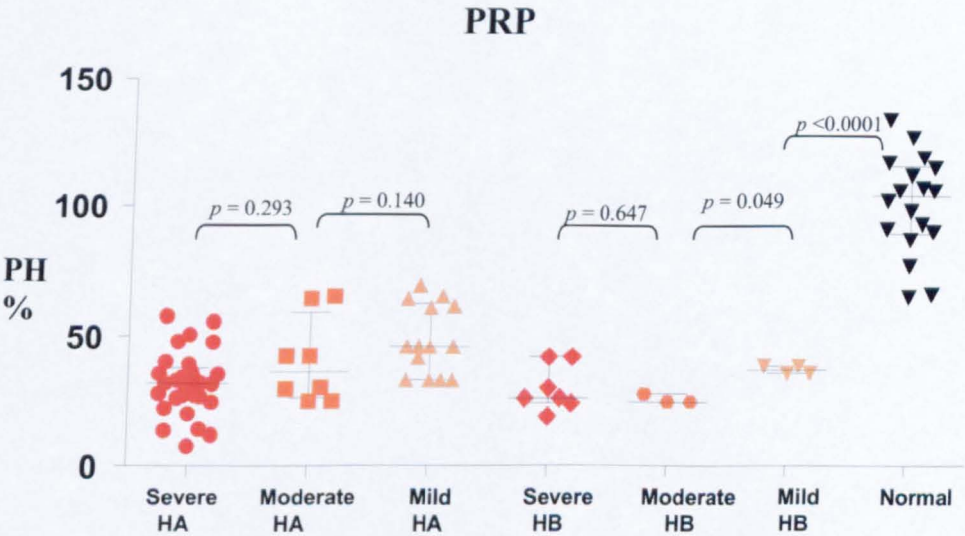


Figure 4.5 Peak height thrombin generation (PRP)
Peak height TGT PRP (expressed as a percentage of the control sample) in severe, moderate and mild HA compared to normal group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

Spearman statistical test was performed to analyse the correlation of PH in all HA and HB patients between different test categories. A significant correlation was found between the PH in PPP and PPP+CTI test categories ($r = 0.873$, $p < 0.0001$) (Table 4.2). However, there were no strong correlations between PH of PPP and PRP ($r = 0.424$, $p = 0.005$), PPP+CTI and PRP+CTI ($r = 0.409$, $p = 0.001$).

Table 4.2 Thrombin generation correlation of test categories

Spearman Correlation	PPP	PPP+CTI	PRP	PRP+CTI
PPP		$r = 0.873$ $p < 0.0001$	$r = 0.424$ $p = 0.005$	$r = 0.294$ $p = 0.045$
PPP+CTI	$r = 0.873$ $p < 0.0001$		$r = 0.452$ $p = 0.0007$	$r = 0.409$ $p = 0.0009$
PRP	$r = 0.424$ $p = 0.005$	$r = 0.452$ $p = 0.0007$		$r = 0.862$ $p < 0.0001$
PRP+CTI	$r = 0.294$ $p = 0.045$	$r = 0.409$ $p = 0.0009$	$r = 0.862$ $p < 0.0001$	

The correlation of PH in-house TGT of all patients between different categories in showed no strong correlation between PPP and PRP. TGT = thrombin generation, PH = peak height, PPP = platelet poor plasma, PRP = platelet rich plasma, CTI = corn trypsin inhibitor.

4.4.1.2. Area under curve

The median and the range (5th - 95th percentiles) of AUC were calculated for the patient groups and normal controls in each test category (Table 4.1). The AUC parameter of patient groups in each test category was compared to the control group, which showed significant decrease compared to normal in all patient groups. Using the Mann-Whitney *U* statistical test a significant difference ($p = 0.003$) was found between the median of control and patient groups in all different categories (Figures 4.6, 4.7, 4.8, and 4.9).

Mann Whitney statistical analysis was performed to compare the median difference of AUC between mild, moderate, and severe HA. Significant difference was found between the AUC of severe and moderate HA patients in PPP+CTI ($p = 0.0237$) and PPP ($p = 0.045$) test categories. However, no significant difference was found between the AUC of mild and moderate groups ($p = 0.536, 0.412$ respectively). In PRP and PRP+CTI categories, no significant differences were found between severe and moderate, or mild and moderate groups (Figures 4.8, and 4.9). In HB groups, a significant difference was found between AUC of moderate and mild HB in PRP test category ($p = 0.049$).

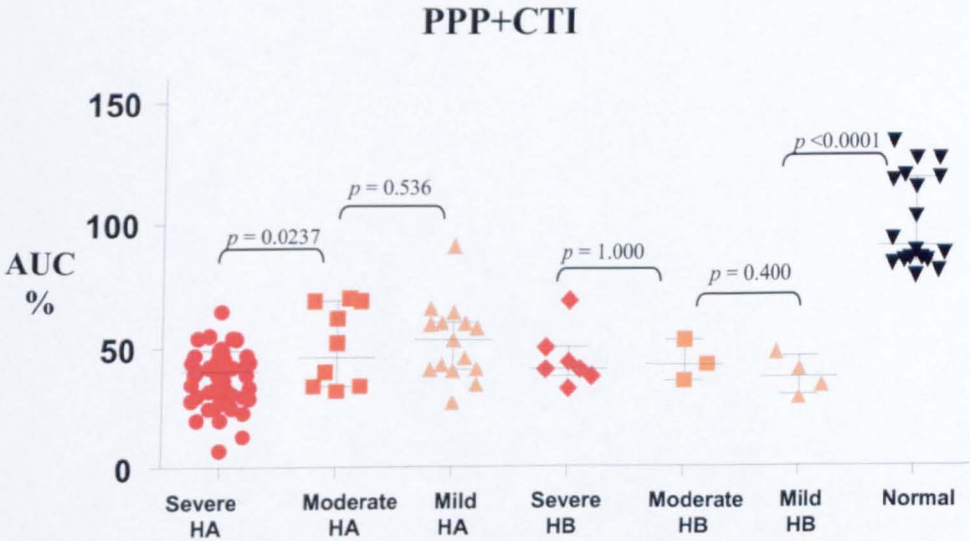


Figure 4.6 Area under the curve thrombin generation in PPP+CTI
The AUC (expressed as a percentage of the control sample) of TGT in PPP+CTI of severe, moderate, mild HA and HB compared to the normal control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

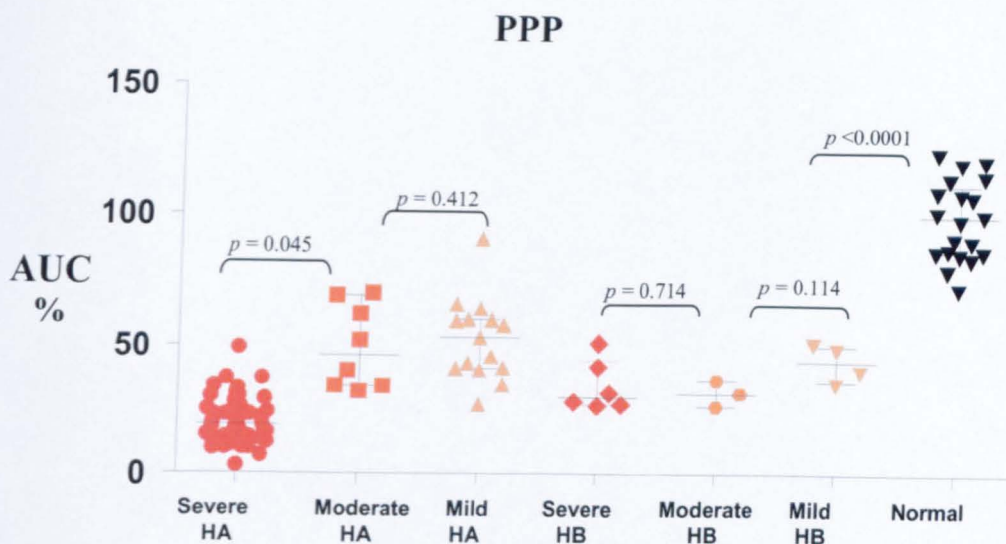


Figure 4.7 Area under the curve thrombin generation in PPP

The AUC (expressed as a percentage of the control sample) of TGT in PPP of severe, moderate, mild HA and HB compared to the normal control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

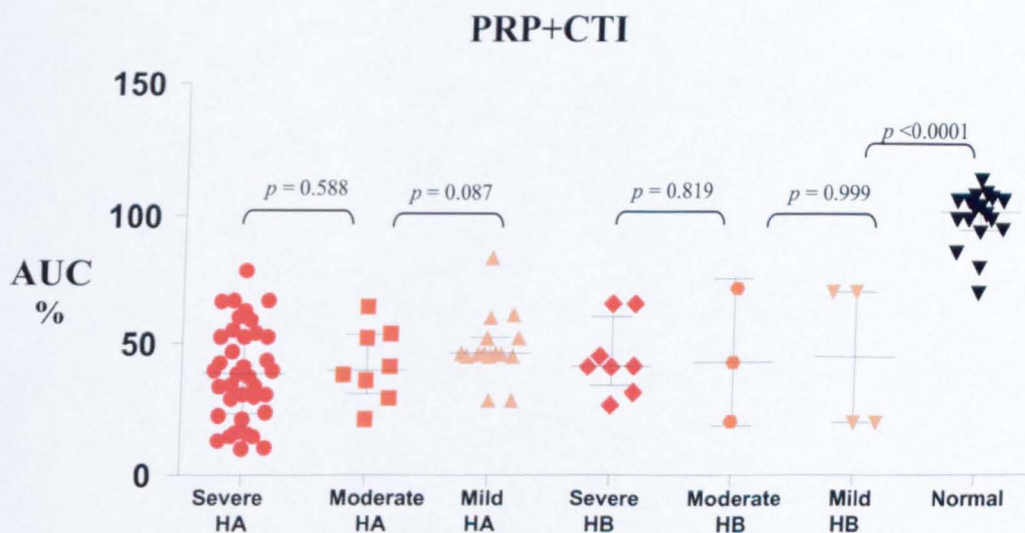


Figure 4.8 Area under the curve thrombin generation in PRP+CTI

The AUC (expressed as a percentage of the control sample) of TGT in PRP+CTI of severe, moderate, mild HA and HB compared to the normal control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

PRP

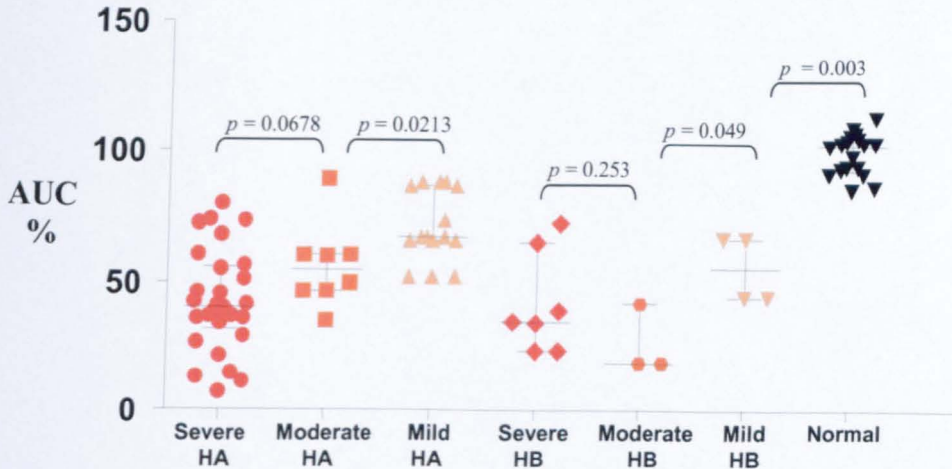


Figure 4.9 Area under the curve thrombin generation in PRP

The AUC (expressed as a percentage of the control sample) of TGT in PRP of severe, moderate, mild HA and HB compared to the normal control group. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

4.4.1.3. Time to peak

The median and range (5th - 95th percentiles) of TP was calculated for the patient groups and normal controls in each test category (Table 4.1). TP parameter of patient groups in each test category was compared to the control group, which showed a significant increase in all patient groups. Using the Mann-Whitney U statistical test a significant difference (the highest $p = 0.042$) was found between the median of control and patient groups in all different test categories (Figures 4.10, 4.11, 4.12, and 4.13).

Mann Whitney statistical analysis was performed to compare the median difference of TP between mild, moderate, and severe HA. Significant difference was found between the TP of severe and moderate HA patients in PPP ($p = 0.0395$) but not in PPP+CTI ($p = 0.1125$) test categories. No significant difference was found between the TP of mild and moderate groups. In PRP and PRP+CTI categories, no significant

differences was found between severe and moderate, or mild and moderate groups (Figures 4.12, and 4.13). In HB groups, no significant difference was found between severe and moderate, or moderate and mild HB subgroups.

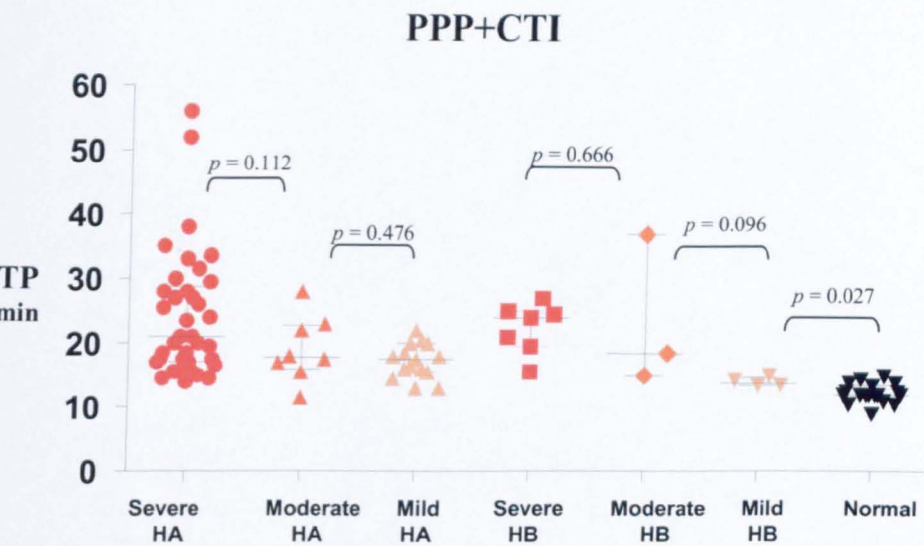


Figure 4.10 Time to peak thrombin generation in PPP+CTI
Time to peak thrombin generation in PPP+CTI of severe, moderate, mild HA and HB compared to normal controls. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the *p* values are for the indicated paired populations)

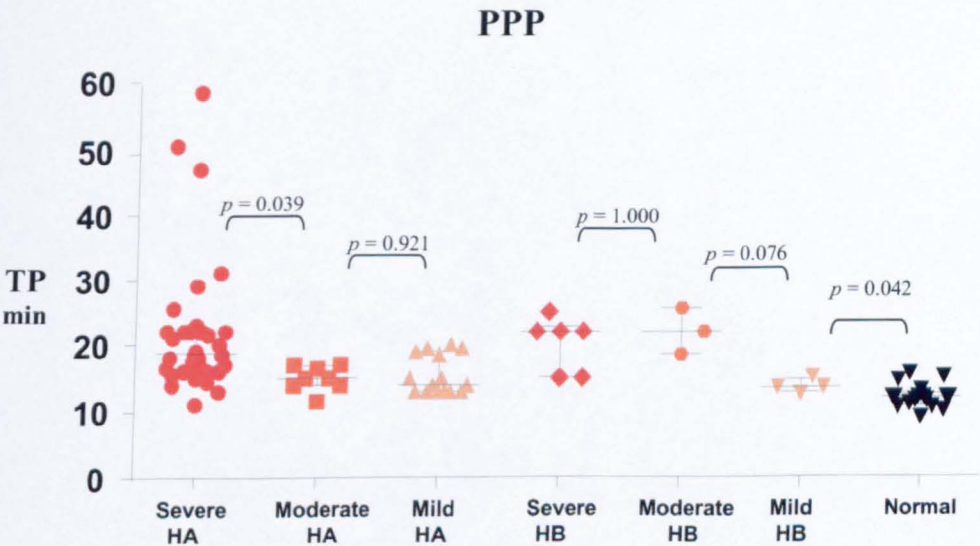


Figure 4.11 Time to peak thrombin generation in PPP
Time to peak thrombin generation in PPP of severe, moderate, mild HA and HB compared to normal controls. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the *p* values are for the indicated paired populations)

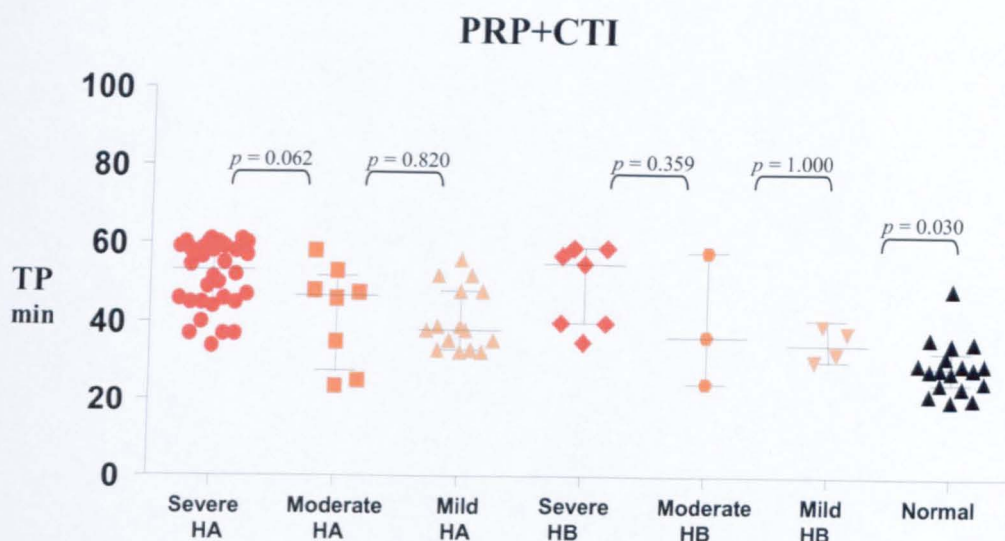


Figure 4.12 Time to peak thrombin generation in PRP+CTI

Time to peak thrombin generation in PRP+CTI of severe, moderate, mild HA and HB compared to normal controls. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

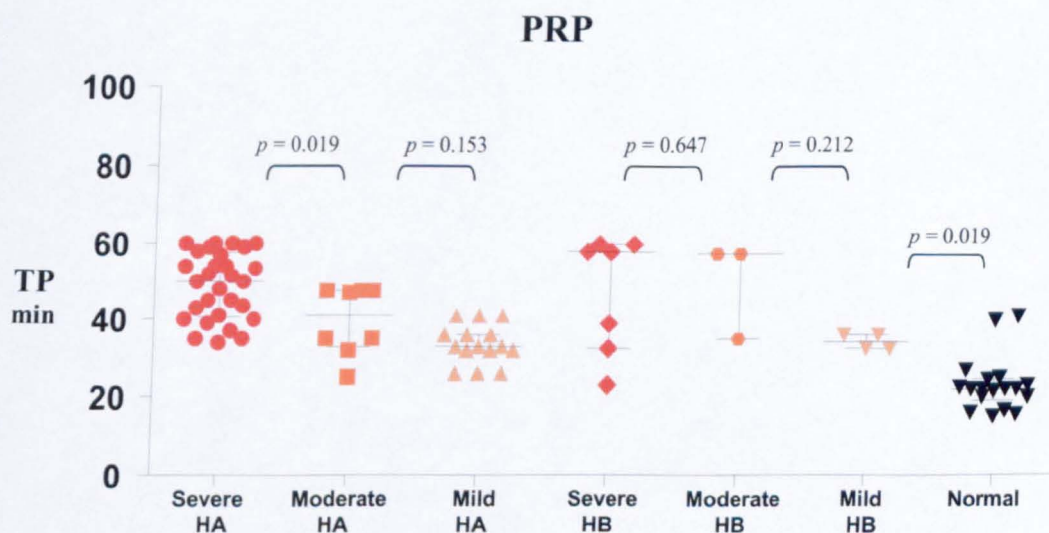


Figure 4.13 Time to peak thrombin generation in PRP

Time to peak thrombin generation in PRP of severe, moderate, mild HA and HB compared to normal controls. Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

4.4.2. Calibrated automated thrombogram®

Calibrated automated thrombogram® (CAT) assay was performed on the PPP+CTI test category only and was compared to the in-house TGT. The CAT parameters were included ETP, Peak thrombin generation, and time to peak (ttPeak). Table 4.3 summarises the results for normal controls and patient groups. The results were converted to percentage of normal in order to compare to the in-house TGT results.

Table 4.3 Calibrated automated thrombogram® (CAT) parameters

CAT	Test Category PPP+CTI	Normal <i>n</i> = 22 Median (5 th - 95 th percentile)	Severe HA <i>n</i> = 36 Median (5 th - 95 th percentile)	Moderate HA <i>n</i> = 8 Median (5 th - 95 th percentile)	Mild HA <i>n</i> = 16 Median (5 th - 95 th percentile)	Severe HB <i>n</i> = 7 Median (5 th - 95 th percentile)	Moderate HB <i>n</i> = 3* Median (5 th - 95 th percentile)	Mild HB <i>n</i> = 3* Median (5 th - 95 th percentile)
ETP	nM.min	1472 (1151-1956)	512 (299-689)	614 (520-844)	602 (473-1136)	437 (366-526)	539 (517-580)	478 (277-772)
	%	95.6 (61.3-156)	30.5 (21-43)	39.5 (33-54)	39 (31-73)	22.5 (17.5-28)	28.3 (24-37)	28 (20-35)
Peak	nM.min ⁻¹	175.5 (115-232)	20 (13.5-54.5)	34.6 (30-48.9)	36.9 (27-90)	24.3 (17-32)	26 (25-28)	27 (16-48)
	%	97 (49-164)	15.5 (9-22)	17.5 (15-25)	19 (14-47)	10 (6-16)	11.3 (10-14)	14 (8-19)
ttPeak	min	9.8 (6.8-13.3)	16 (14.6-18.8)	14.3 (12-17)	15.3 (11-18)	14.9 (12-19)	20 (18.5-22)	15.8 (12-20)

The median and the range (5th - 95th percentiles) CAT parameters were calculated in control group and Haemophilia patients in PPP+CTI category. CAT = calibrated automated thrombogram, ETP = endogenous thrombin potential, Peak = maximum peak height thrombin generation, ttPeak = time to maximum peak height thrombin generation, PPP = platelet poor plasma, CTI = corn trypsin inhibitor, HA = haemophilia A, HB = haemophilia B.

*where the number of samples was not statistically significant, minimum and maximum values were reported

Mann-Whitney statistical analysis showed no significant difference between the Peak ($p = 0.0928$), ETP ($p = 0.720$), or ttPeak ($p = 0.817$) of moderate and mild HA individuals. There were also no significant differences between the Peak ($p = 0.066$) or ttPeak ($p = 0.318$) of moderate and severe HA. However there was significant difference between the medians of ETP in the severe and moderate HA ($p = 0.0405$).

Using the Spearman statistical test, in-house TGT parameters of all HA and HB patients ($n = 73$) in the PPP+CTI test category were compared to CAT parameters (Table 4.4) (r value of > 0.5 and p value of < 0.05 considered to be strong and

significant correlation). A relatively strong correlation was observed between PH and Peak thrombin generation ($r = 0.635$, $p < 0.0001$). In contrast, AUC had a lesser correlation with ETP ($r = 0.538$, $p = 0.001$) and TP had a weak correlation but significant with the ttPeak ($r = 0.254$, $p = 0.005$).

Table 4.4 Comparison of in-house TGT and CAT

<div style="text-align: center;">TGT CAT</div>	AUC%	PH%	TP
ETP%	$r = 0.538$ $p = 0.001$	$r = 0.595$ $p < 0.0001$	$r = -0.282$ $p = 0.01$
Peak%	$r = 0.577$ $p = 0.001$	$r = 0.635$ $p < 0.0001$	$r = -0.334$ $p = 0.201$
ttPeak	$r = -0.099$ $p = 0.520$	$r = -0.135$ $p = 0.006$	$r = 0.254$ $p = 0.005$

A strong and significant correlation was observed between PH and Peak when TGT parameters were compared to CAT parameters. AUC and ETP had a less significant correlation in comparison with PH and Peak, whilst TP and ttPeak a weak but significant correlation ($n = 73$). CAT = calibrated automated thrombogram, ETP = endogenous thrombin potential, Peak = maximum peak height thrombin generation, ttPeak = time to maximum peak height thrombin generation, TGT = in-house thrombin generation test, AUC = area under the thrombin generation curve, PH = peak height thrombin generation, TP = time to peak height thrombin generation.

ROCs were calculated for all parameters in each test category in mild HA only (Table 4.5). The PH was the most sensitive parameter of TGT in PRP+CTI test category with sensitivity of 100% and specificity of 94% followed by PPP+CTI (sensitivity = 94%, specificity = 94%) to distinguish between hypocoagulability and normal states. The sensitivity and the specificity analysis in this section was meant to be used for evaluation of diagnostic power of TGT. The ROC was used demonstrate if parameters of the TGT or CAT have a potential to discriminate between two groups of normal and abnormal in terms of their thrombin generation capacity. The results showed that

the use of CTI would improve the sensitivity and the specificity of the test. The Peak was the most sensitive parameter of the CAT but less sensitive (92%) and specific (90%) than the PH in TGT. The sensitivity and specificity of AUC was relatively low when compared to the PH parameter. The sensitivity and specificity of TP was lowest among other parameters of TGT.

Table 4.5 Thrombin generation sensitivity and specificity in all test categories

TGT	Test Category	ROC (Mild HA patients)		
		Sensitivity %	Specificity %	Likelihood Ratio
PH %	PPP	80	94	13.6
	PPP+CTI	94	94	16.9
	PRP	83	94	15.0
	PRP+CTI	100	94	18.0
AUC %	PPP	80	94	13.6
	PPP+CTI	88	94	15.8
	PRP	50	94	9.0
	PRP+CTI	79	94	14.0
TP min	PPP	40	94	6.8
	PPP+CTI	75	94	13.5
	PRP	60	94	10.8
	PRP+CTI	56	94	10.0
ETP %	PPP+CTI	69	95	13.9
Peak %	PPP+CTI	92	90	9.2
ttPeak min	PPP+CTI	62	95	12.3

Sensitivity and specificity of TGT and CAT for each test category were analysed. PH in PRP+CTI was the most sensitive parameter of in-house TGT. ROC = receiver operating characteristic curve, CAT = calibrated automated thrombogram, ETP = endogenous thrombin potential, Peak = maximum peak height thrombin generation, ttPeak = time to maximum peak height thrombin generation, TGT = in-house thrombin generation test, AUC = area under the thrombin generation curve in TGT, PH = peak height thrombin generation in TGT, TP = time to peak height thrombin generation in TGT, PPP = platelet poor plasma, PRP = platelet rich plasma, CTI = corn trypsin inhibitor.

The sensitivity and specificity was also calculated for the most sensitive parameters of in-house TGT and CAT for all patients ($n = 73$) (Table 4.6). The in-house PH in PRP+CTI category showed the highest sensitivity and specificity (100%, and 94% respectively).

Table 4.6 Sensitivity and specificity for all patients

		Sensitivity %	Specificity %	Likelihood ratio
PH	PRP+CTI	100	94.4	18.0
PH	PPP+CTI	99.0	94.4	17.8
Peak	PPP+CTI	97.7	90.0	9.8

Sensitivity and specificity of TGT for peak height of TGT and CAT were analysed. PH in PRP+CTI was the most sensitive parameter of in-house TGT. TGT = thrombin generation test, PH = TGT, PH = peak height thrombin generation in TGT, Peak = peak height in calibrated automated thrombogram[®], PPP = platelet poor plasma, PRP = platelet rich plasma, CTI = corn trypsin inhibitor.

4.5. Discussion

Since the modification and simplification of TGT by Hemker *et al.* its clinical application has been increased in recent years. Although the utility and reliability of TGT in various clinical scenarios is yet to be proven, there is growing interest in finding a test which can measure the generation of thrombin, the central player of haemostasis and thrombosis. Several studies support the value of thrombin generation in detecting hyper- and hypocoagulable states (Beltran-Miranda *et al.*, 2005; Dargaud *et al.*, 2005; Dargaud *et al.*, 2007; Dargaud *et al.*, 2010; Hemker *et al.*, 2006; Lewis *et al.*, 2007; van Veen *et al.*, 2009).

In this study, thrombin generation in a cohort of haemophilia A and B patients was evaluated under four different test categories. The use of CTI, to inhibit the contact pathway, was also investigated by comparing samples with and without CTI in PPP

and PRP. Under each condition, the PH, TP and the AUC parameters of thrombin generation were measured and compared to the group of healthy individuals.

The results showed a decrease in PH and AUC, and a prolongation of TP in all patient groups and all test categories when compared to the normal group ($P < 0.0001$). In the severe HA group the PH ranged 6.6-31.3% in the PPP+CTI category with an SD of 7%, and in PPP category the PH ranged 6.7-39.5% with an SD of 8.1%. This suggests that the use of CTI reduced the variability confirming findings by other researchers (Luddington & Baglin, 2004) that some of the variations are the result of sample activation prior to or during sample preparation in the PPP or PRP categories when no CTI was used.

The AUC results showed a high within-group variation in the severe HA group with a range of 12.4-56.1% (SD = 12.41%) in PPP+CTI and 14.9-61.2% (SD = 11.99%) in PPP categories. These wide ranges and high SD confirm the earlier reports of heterogeneity of thrombin generation in haemophilia patients (Lewis *et al.*, 2007; van Veen *et al.*, 2009) suggesting that some of these variations with similar levels of FVIII may be related to other coagulation factors and all variations cannot be attributed to cellular components.

The heterogeneity of thrombin generation in severe HA group was greater in PRP+CTI (PH ranged 9.3-57.6%) and PRP (PH ranged 9.5-56.9%) when compared to PPP+CTI (PH ranged 6.4-31.5%) and PPP (PH ranged 6.7-39.5%) test categories. This higher variation may be partly related to the source of phospholipids, which in PRP is the individual's own platelets, unlike synthetic phospholipids used in PPP

TGT. The heterogeneity of platelets has been alluded to by various experts as a potential basis for the heterogeneity of the clinical phenotype (Dargaud, 2006; Yee, 2006). Indeed very early coagulation studies relied on patients own platelets as a source of phospholipids causing marked inter-individual variability (Buckwalter *et al.*, 1949). To improve reproducibility and decrease variability, exogenous phospholipid has been used for the last few decades with improvement in the reproducibility for the various coagulation tests (Monroe *et al.*, 2002).

The low concentration of TF (0.5 pM) in PRP may also increase the variability of the results. Lewis et al. (2007) reported a wide variability of thrombin generation of severe HA patients in PRP+CTI samples. They could only report the initial rate of thrombin generation due to a too low TF concentration (0.3 pM) for initiation of the coagulation. As a result, the ETP and Peak thrombin generation could not be calculated in their study. In this study the final concentration of TF for PRP TGT was 0.5 pM, which enabled us to obtain the AUC results within the running time of the test.

The median PH for generated thrombin in PRP+CTI and PRP was higher than the median PH in PPP+CTI ($p = 0.0001$) and PPP ($p = 0.0164$) in the severe HA group, despite a low concentration of TF. The effect of VWF cannot be ignored as some individuals had high levels of VWF. A recent study has shown that VWF in addition to its function in platelet adhesion and as the carrier of FVIII, is also required for thrombin generation in PRP via a mechanism in which fibrin and platelet receptor GP1b are involved (Faber *et al.*, 2003). Therefore, thrombin generation in PRP is

dependent upon platelet function (Dargaud *et al.*, 2006a), VWF level, and the concentration of FVIII/FIX in haemophilia A/B.

Significant difference was found between the median PH of severe and moderate HA patients in PPP and PPP+CTI test categories ($p = 0.0017$, and 0.0001 respectively). The results suggest that thrombin generation is dependent upon the level of FVIII between severe (FVIII < 1.0 IU/dL) and moderate (FVIII = 1-5.0 IU/dL). However, no significant difference was found between the PH of moderate and mild groups ($p = 0.412$, 0.536 respectively), although the sample size in these groups was not statistically significant ($n = 8$ and 15 , respectively).

The analysis of the sensitivity and specificity of the TGT parameters showed the TGT can be used to distinguish between normal and abnormal in terms of their thrombin generation potential. The likelihood ratio was significantly higher in the PH parameter of in-house TGT in PPP+CTI (likelihood ratio = 17.8) when compared to the Peak parameter of the CAT in the same test category (likelihood ratio = 9.8).

In PPP+CTI test category, there was a relatively strong correlation between PH of in-house TGT and Peak height of CAT ($r = 0.635$, $p = <0.0001$), and to lesser extent between AUC and ETP ($r = 0.538$, $p = 0.001$). However, there was a weak correlation between the TP of in-house TGT and ttPeak of CAT ($r = 0.254$, $p = 0.005$). Although the methods are similar in principle such discrepancies are not uncommon. In recent multicentre studies the use of different TF and PL sources was shown to produce a large variability in thrombin generation testing (Dargaud *et al.*, 2010; Gerotziafas *et al.*, 2005). In our study the TF source and the dilution factor was similar to the CAT

starting reagent, but the CAT reagent was supplied in a lyophilised form in which the phospholipids are also incorporated into the reagent. In contrast, in the in-house method, the tissue factor was freshly made, diluted and just prior to the testing, freshly made phospholipids were added to the plasma sample. This may explain the precision and accuracy of the in-house TGT which is revealed in the ROC analysis in which the in-house TGT results were more sensitive and specific to distinguish between normal and abnormal thrombin generation potential, when compared to the CAT analyser.

4.6. Conclusion

TGT appears to be a reliable test to exclude individuals with lower than normal coagulation FVIII or FIX levels. In particular the likelihood ratio of PH makes this parameter ideal for investigation of hypocoagulability. The use of CTI to eliminate the interference of the contact system was also shown to play an important part in improving the accuracy and precision of thrombin generation. Although crossovers of results were observed between populations of severe, moderate, and mild groups in different parameters of TGT, but in general, statistically the differences were significant. For those who did not fall into the relevant severity group, however, this may indicate that the severity classification of haemophilia by TGT may not necessarily follow the same severity classification with clotting factor assay. The global assays may reflect the effect of other modifying elements which may have positive or negative

The heterogeneity of thrombin generation, in particular in severe HA and HB, was also demonstrated in this study, suggesting that the rate and amount of thrombin generation are not entirely dependent on the small amounts of FVIII or IX in this group of patients.

Other modifiers of thrombin generation are discussed in the following chapters.

Chapter 5 **Clot waveform analysis**

5.1. Introduction

The accurate assay of clotting factor levels is critical in the diagnosis and management of patients with clotting factor deficiencies. Measurement of FVIII or FIX activity is generally performed by one stage clotting based assays, or by amidolytic assays using chromogenic substrates. The lower assay limit is often affected by the quality of the deficient plasma, activating reagent, type of standard, and other analytical variables. As a result, the lower detection limit of conventional assays, such as one-stage APTT based clotting assay for determination of FVIII or FIX levels, is generally 1.0-2.0 IU/dL (Hardisty & Macpherson, 1962). Therefore, it is difficult to diagnose the true severity of haemophilia A or B by conventional assays when the level of FVIII or FIX is measured as 1.0 IU/dL or less (Shima *et al.*, 2008).

During the performance of routine clotting assays such as APTT and PT, it is possible to obtain a continuous measurement of changes in light transmittance that occur as the test plasma sample clots. The resultant photo-optical data profiles obtained by continuous monitoring are called clot waveforms. The waveform is mathematically processed by a software algorithm to derive several parameters such as coagulation velocity and acceleration (Downey *et al.*, 1997).

Using this approach, Shima *et al.* (2002) observed that the APTT clot waveform analysis on a number of patients with severe HA was different from patient to patient despite the fact that all had FVIII:C level of < 1.0 IU/dL. In their experiment, samples of FVIII deficient plasma were prepared to which serially diluted recombinant FVIII

was added to provide an array of contrived FVIII deficient plasma samples ranging from zero to 1.0 IU/dL (0, 0.1, 0.2, 0.3, 0.5, 0.75, and 1.0 IU/dL final concentration FVIII). One-stage clotting assay and clot waveform analysis were performed on the diluted samples. FVIII level in these series was closely correlated with the minimum value of the second derivative (Min2) of the APTT waveform. Min2 is the measure of the acceleration of change in optical transmission at the initiation of coagulation.

This data suggests that the APTT is variably affected by other plasma factors (Shima *et al.*, 2008). Other studies have also indicated that clot waveform analysis and especially Min2 values may have greater discriminatory power in assessing low clotting factor activity and may provide additional information in these patients (Shima *et al.*, 2002). Thus clot waveform analysis may be useful for the investigation of the clinical phenotype of individual patients and their response to therapy.

This chapter describes the clot waveform analysis on haemophilia A and B individuals who participated in this study. The APTT test was performed on the MDA[®] coagulometer (Organon Teknika, Cambridge, UK) in the Haemostasis laboratory, Addenbrooke's Hospital, Cambridge, UK with kind permission of Dr Roger Luddington. The waveform was mathematically processed by a software algorithm, provided with the MDA[®], to derive two relevant parameters, Min1 (maximum coagulation velocity, %T/s) and Min2 (maximum coagulation acceleration, %T/s²).

5.2. Study subjects

Ninety seven patients were consented for the study, and clot waveform analysis were performed on 73 individuals with haemophilia who were included in the study. These comprised 36 severe, eight moderate, 15 mild HA, and seven severe, three moderate, and four mild HB, aged between 18 and 90 years (mean 43, median 41 years). The control group comprised 22 healthy male individuals aged between 22 and 62 (mean 40, median 39 years). Patients on prophylaxis or on demand treatment were asked to omit treatment for a minimum of 72 h to allow the FVIII or FIX level to return to baseline level (All severe haemophilia individuals samples included in the statistical analysis had FVIII:C or FIX:C levels < 1.0 IU/dL, after wash-out period (sections 2.1.1 and 2.1.2) as measured by the one stage clotting assay). Those who required treatment were excluded from study. The study was approved by the ethics committee of the Royal Free Hospital, and informed consent was obtained from patients and healthy individuals.

5.3. Results

The APTT, Min1 (velocity) and Min2 (acceleration) parameters (section 1.5) of clot waveform analysis were extracted from software on the MDA[®] coagulometer. One-stage clotting factor assay was performed on all patients and control groups. The results showed nonparametric distributions, therefore median and the ranges (based on 5th- 95th percentiles) for Min1 and Min2 were calculated for the patient and normal control groups (Table 5.1). The results were also normalised and expressed in percentage of mean normal (control group). The results showed a significant decrease of Min1 and Min2 in all patient groups. The CV for Min1, Min2 and APTT in severe

HA group were 42%, 52%, and 23% respectively. In severe HB group the CV for the same parameters were 37%, 50%, and 24% respectively. Using the Mann-Whitney *U* statistical test a significant difference ($p < 0.0001$) was found between the median of control and patient groups in all different categories (Figures 5.1, 5.2).

Table 5.1 Clot waveform analysis on haemophilia A and B, and control group.

Clot Wave form	Normal <i>n</i> = 22 Median (5 th - 95 th percentile)	Severe HA <i>n</i> = 36 Median (5 th - 95 th percentile)	Moderate HA <i>n</i> = 8 Median (5 th - 95 th percentile)	Mild HA <i>n</i> = 15 Median (5 th - 95 th percentile)	Severe HB <i>n</i> = 7 Median (5 th - 95 th percentile)	Moderate HB <i>n</i> = 3* Median (5 th - 95 th percentile)	Mild HB <i>n</i> = 4* Median (5 th - 95 th percentile)
Min1 %T/s	6.616 (5.415-8.668)	1.500 (0.564-3.14)	2.519 (1.392-4.275)	3.542 (2.154-5.571)	1.737 (1.22-3.035)	3.126 (2.441-4.737)	3.525 (3.077-4.522)
Min1 %	98.0 (80-129)	22.0 (8-47)	37.4 (21-63)	53.0 (32-83)	26.0 (18-46)	47.0 (37-72)	53.0 (47-68)
Min2 %T/s ²	0.584 (0.508-0.757)	0.113 (0.028-0.287)	0.25 (0.12-0.45)	0.373 (0.185-0.593)	0.136 (0.09-0.318)	0.328 (0.258-0.508)	0.357 (0.295-0.484)
Min2 %	97 (84-125)	19 (5-48)	41 (20-74)	62 (31-98)	23 (15-54)	56 (44-87)	61 (51-83)
APTT s	29.0 (24-36)	70.2 (55.5-111.6)	53.1 (45-66.1)	42.2 (35.8-75.9)	58.3 (47-92.7)	50.0 (43.5-58.3)	48.5 (34.4-59.6)
FVIII/ FIX IU/dL	100 (50-150)	< 1	3 (1-5)	11 (5-48)	< 1	2 (2-4)	5 (5-13)

The median and the range (5th- 95th percentiles) Clot waveform parameters, APTT, and FVIII/FIX levels were calculated in patient and control groups. Min1 = minimum value of first derivative (coagulation velocity, % change in transmittance (T)/second) Min2 = minimum value of second derivative (coagulation acceleration, % change in transmittance (T)/second²)

*where the number of samples was not statistically significant, minimum and maximum values were reported

Mann Whitney statistical analysis was performed to compare the median difference of Min1 and Min2 between mild, moderate, and severe HA. Significant difference was found between the Min1 of severe and moderate HA patients ($p = 0.003$), and the Min2 of severe and moderate HA ($p = 0.001$). No significant difference was found between the Min1 or Min2 of mild and moderate HA groups ($p = 0.057$, $p = 0.084$ respectively). In the HB group, a significant difference was found between severe and moderate HB for Min1 ($p = 0.017$) and Min2 ($p = 0.017$) however, no significant difference was found between moderate and mild HB for neither Min1 ($p = 0.857$) nor Min2 ($p = 0.990$).

Clot Waveform

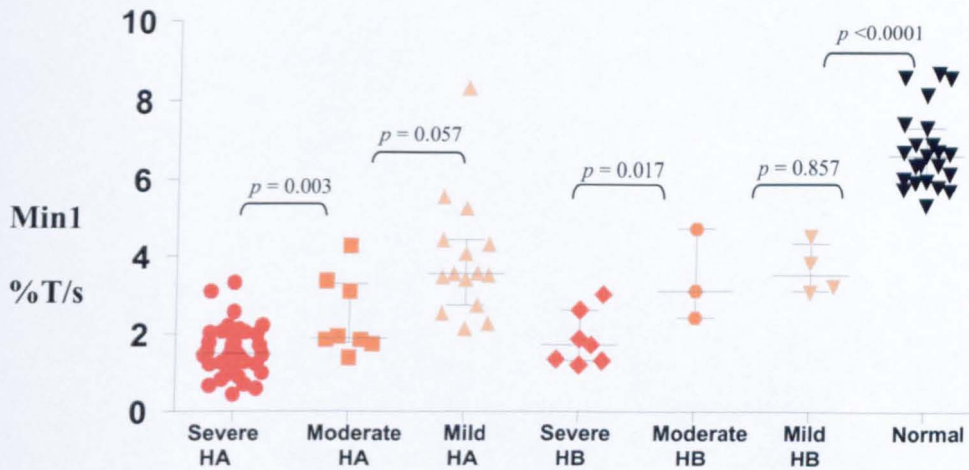


Figure 5.1 Clot waveform analysis (Min1)

Significant differences were found between severe and moderate HA ($p = 0.003$), but not between moderate and mild HA ($p = 0.057$). In HB, a significant difference was found between severe and moderate HB ($p = 0.017$), but no significant difference between moderate and mild HB ($p = 0.857$). Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

Clot Waveform

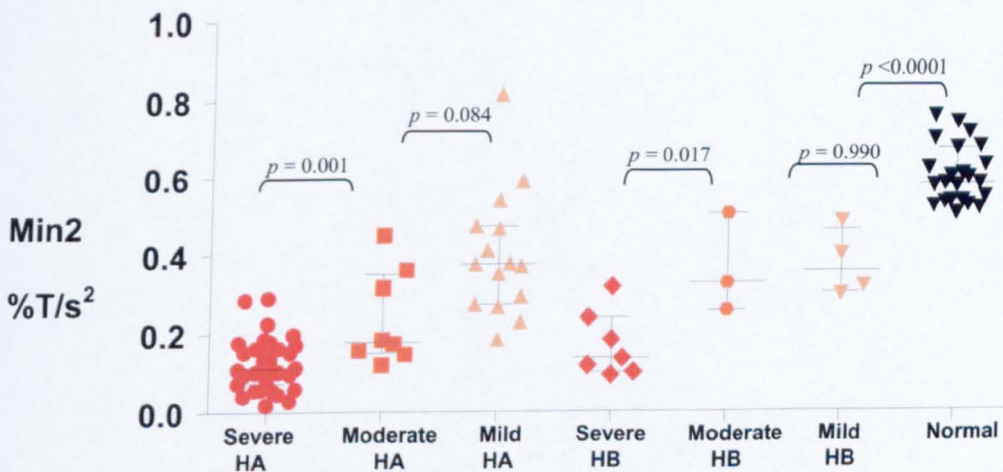


Figure 5.2 Clot waveform analysis (Min2)

Significant differences were found between severe and moderate HA ($p = 0.001$), but not between moderate and mild HA ($p = 0.084$). In HB, a significant difference was found between severe and moderate HB ($p = 0.017$), but no significant difference between moderate and mild HB ($p = 0.990$).

Median (middle line) with inter-quartile range (error bars) illustrated in each group. (the p values are for the indicated paired populations)

Statistical analysis by Spearman correlation was performed to compare the clot waveform parameters, FVIII level and APTT of moderate and mild HA (combined) (Table 5.2). Significant correlation was found between Min1 and FVIII level ($r = 0.786, p < 0.0001$), however, the correlation between Min1 and APTT ($r = -0.611, p < 0.0001$) was not as strong as the correlation with FVIII level. Min2 correlation with FVIII level was also significantly strong ($r = 0.759, p < 0.0001$) and to lesser extent with APTT ($r = -0.658, p < 0.0001$). The correlation between FVIII level and the APTT was moderately strong ($r = -0.513, p = 0.001$).

Table 5.2 Clot waveform correlation with FVIII and APTT

Clot waveform analysis	Min1	Min2	APTT	FVIII
Min1 $n = 23$		$r = 0.985$ $p < 0.0001$	$r = -0.611$ $p < 0.0001$	$r = 0.786$ $p < 0.0001$
Min2 $n = 23$	$r = 0.985$ $p < 0.0001$		$r = -0.658$ $p < 0.0001$	$r = 0.759$ $p < 0.0001$
APTT $n = 23$	$r = -0.611$ $p < 0.0001$	$r = -0.658$ $p < 0.0001$		$r = -0.513$ $p = 0.001$
FVIII $n = 23$	$r = 0.786$ $p < 0.0001$	$r = 0.759$ $p < 0.0001$	$r = -0.513$ $p = 0.001$	

Spearman correlation was performed to analyse the relation between clot waveform parameter, FVIII, and APTT of combined moderate and mild HA ($n = 23$). Min1 = minimum value of first derivative (coagulation velocity), Min2 = minimum value of second derivative (coagulation acceleration).

To illustrate a better picture of correlation between the clot waveform parameters, APTT, and the FVIII level, linear regression analysis was performed between combined moderate and mild HA group Min1/Min2 and FVIII (Figures 5.3 and 5.4) and APTT (Figures 5.5 and 5.6).

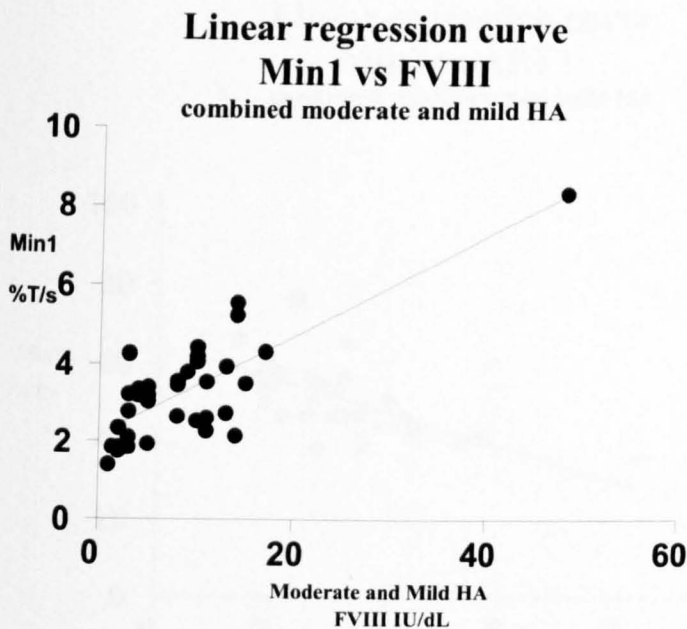


Figure 5.3 Clot waveform linear regression curve (Min1 vs FVIII)
Linear regression curve of Min1 clot wave form on moderate and mild HA ($r^2 = 0.624$) (the outlier FVIII = 48 IU/dL)

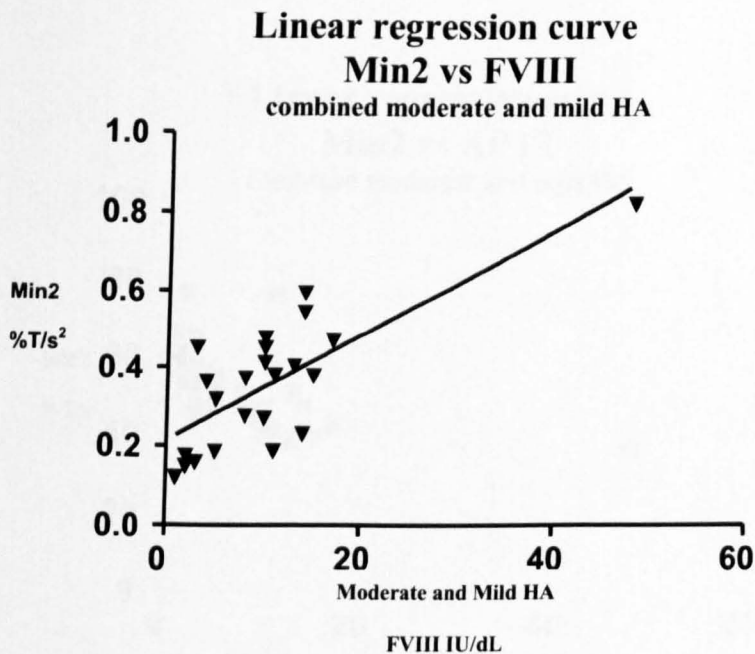


Figure 5.4 Clot waveform linear regression in combined moderate and mild (Min2 vs FVIII)
Linear regression curve of Min2 clot wave form on combined moderate and mild HA ($r^2 = 0.568$) (the outlier FVIII = 48 IU/dL).

Linear regression curve
Min2 vs APTT
combined moderate and mild HA

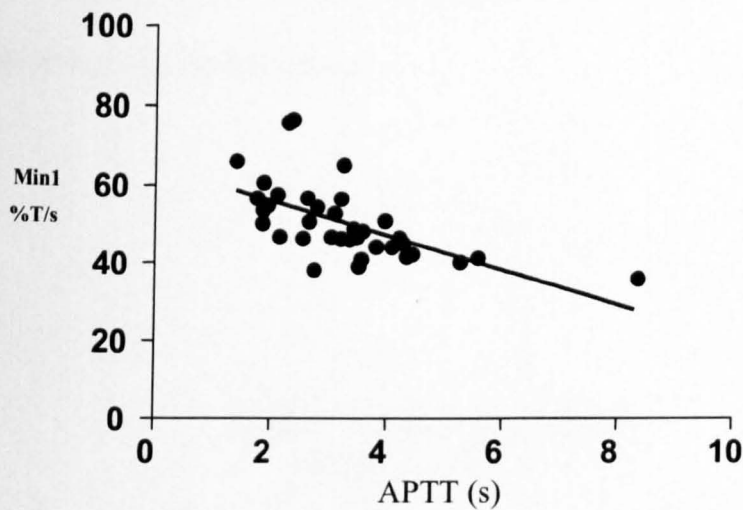


Figure 5.5 Clot waveform linear regression in combined moderate and mild HA (Min1 vs APTT)

Linear regression curve of APTT clot wave form on moderate and mild HA ($r^2 = 0.374$).

Linear regression curve
Min2 vs APTT
combined moderate and mild HA

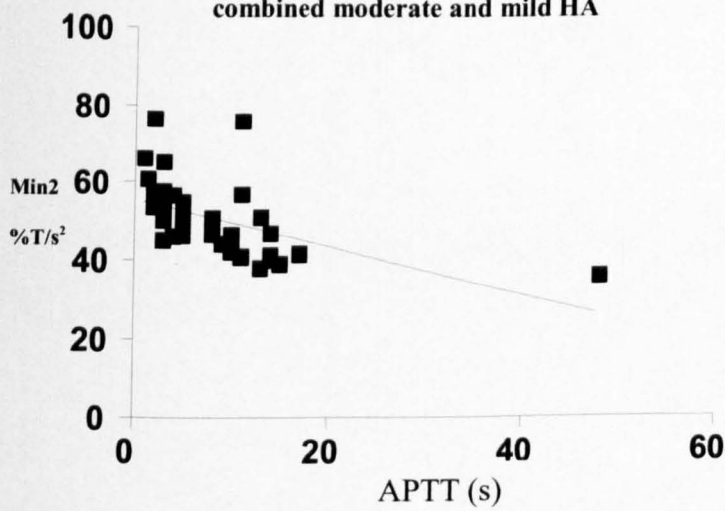


Figure 5.6 Clot waveform linear regression in combined moderate and mild HA (Min2 vs APTT)

Linear regression curve of APTT clot wave form on moderate and mild HA ($r^2 = 0.229$).

In the severe HA group, because FVIII < 1.0 IU/dL correlation could not be performed, however, linear regression analysis was performed between Min1/Min2 and APTT of severe HA group (Figures 5.7 and 5.8). The r^2 was 0.274 and 0.332 for Min1 and Min2 respectively.

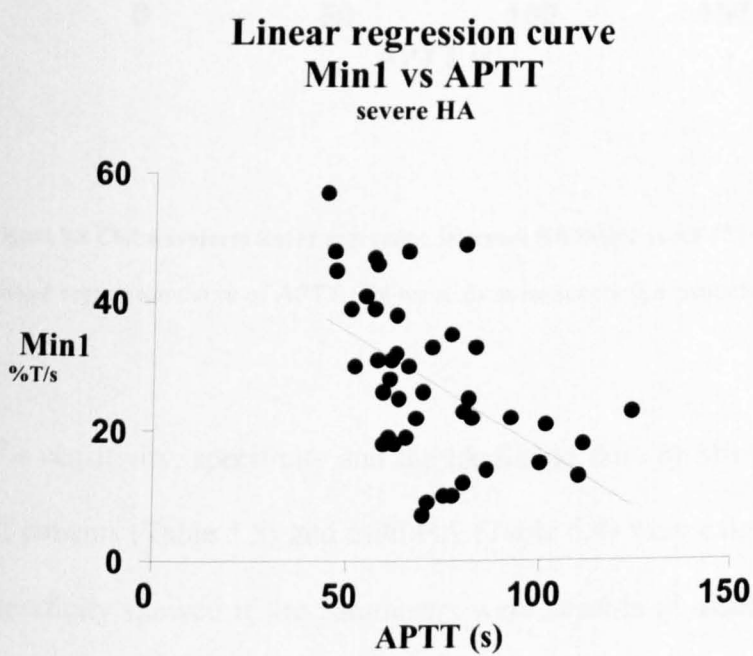


Figure 5.7 Clot waveform linear regression in severe HA (Min1 vs APTT)

Linear regression curve of APTT clot wave form on severe HA patients ($r^2 = 0.274$).

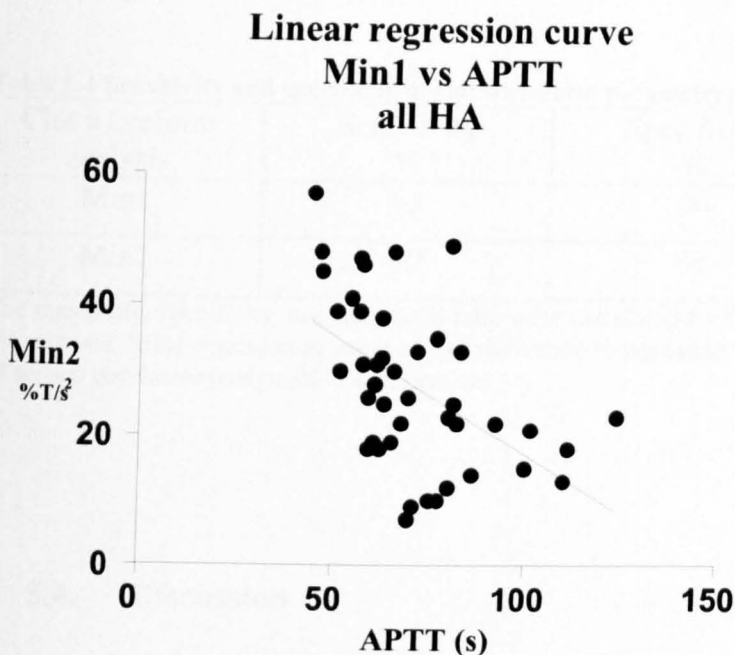


Figure 5.8 Clot waveform linear regression in severe HA (Min2 vs APTT)

Linear regression curve of APTT clot wave form on severe HA patients ($r^2 = 0.332$).

The sensitivity, specificity and the likelihood ratio of Min1 and Min2 parameters for all patients (Table 5.3) and mild HA (Table 5.4) were calculated. The sensitivity and specificity showed if the parameters were capable of distinguishing between normal and abnormal coagulation velocity and acceleration.

Table 5.3 Sensitivity and specificity of clot waveform parameters for all patients

Clot waveform analysis	Sensitivity %	Specificity %	Likelihood Ratio
Min1	99	96	22.7
Min2	97	96	22.2

The sensitivity, specificity, and likelihood ratio were calculated for Clot waveform parameters of all patients. Min1 = minimum value of first derivative (coagulation velocity) Min2 = minimum value of second derivative (coagulation acceleration).

Table 5.4 Sensitivity and specificity of clot waveform parameters mild HA patients

Clot waveform analysis	Sensitivity %	Specificity %	Likelihood Ratio
Min1	93	96	21.5
Min2	80	96	18.4

The sensitivity, specificity, and likelihood ratio were calculated for Clot waveform parameters of Mild HA patients. Min1 = minimum value of first derivative (coagulation velocity), Min2 = minimum value of second derivative (coagulation acceleration).

5.4. Discussion

The clinical significance of detecting levels of FVIII/FIX activities of < 1.0 IU/dL is very high as the individuals with such levels are at greatest risk of bleeding. However, not all individuals with severe haemophilia experience the same clinical phenotype. It is known from clinical experience that approximately 10-15% of severe haemophilia patients, despite similar FVIII/FIX plasma levels of < 1.0 IU/dL, can exhibit a milder bleeding phenotype (Bolton-Maggs & Pasi, 2003).

The clot waveform analysis performed in this study showed that such heterogeneity exists among the severe HA and HB individuals, and it was more pronounced in Min1 and Min2 parameters when compared to the APTT. The broad range of Min1 (5th – 95th percentile = 8-47%) and Min2 (5th – 95th percentile = 5-48%) in severe HA and HB individuals (18-46%, and 15-54% respectively) demonstrate that categorising all severe haemophilia individuals under the same “ < 1.0 IU/dL” activity level of their relevant factor may not reflect their clinical phenotype manifestation.

The APTT, as well as the one-stage clotting assay, measures the endpoint, which is the clotting time of a citrated plasma sample. This is the point at which coagulation is initiated and the first visible fibrin strands appear. In contrast, Min1 represents the velocity of the reaction in the process of clot formation and Min2 is a measurement of acceleration of change in light transmission, whereas the APTT or one-stage clotting assay is the time at which the fibrin is formed. The data in this chapter confirms previous experiment by Shima *et al.* (2002), that the correlation of Min1 and Min2 with the level of the FVIII is more significant than the correlation of APTT and the FVIII.

The coefficient of determination (r^2) for Min1 and FVIII was 0.624, which shows that more than 62% of variance in Min1 axis can be explained by variance of FVIII activity level axis. The r^2 for Min2 was 0.568 which also shows that 57% of the variance in the Min2 axis can be explained by variance of FVIII activity levels axis. Therefore, the APTT was shown to be a weak predictor to explain the variance of FVIII with r^2 of 0.229. This may indicate that the time to clot formation may be affected by other modifying or interfering factors, whereas such influence on the rate and the acceleration of clot formation may be less effectual than the deficient factor.

The sensitivity and specificity of clot waveform analysis showed that the Min1 and Min2 could be used as discriminatory parameters in assessing the severity of HA and HB and for monitoring haemophilia patients.

5.5. Conclusion

The result of this study demonstrates that patients generally assigned to having FVIII/FIX levels of < 1.0 IU/dL may have variable concentrations below that level which cannot be reliably measured by conventional methods. The clot waveform parameters, Min1 and Min2, appear to be reliable predictors of FVIII/IX levels, based on their coefficient of determination, and can be used in cases where the level of < 1.0 IU/dL is interested such as gene therapy monitoring, or monitoring treatment, on known HA or HB patients and in an emergency situation i.e. out of hours, where the technical support is limited. Although these parameters are sensitive in distinguishing abnormal results from normal, they are not specific to a particular disorder. These parameters are simple to extract from the raw data, fast to obtain, and cost effective.

The future studies could include haemophilia C as it has even greater phenotype heterogeneity compared to FXI activity level.

Chapter 6 **Variation of clinical phenotype in haemophilia**

6.1. Introduction

The variability of clinical phenotype among severe haemophilia patients has been documented in several studies (Aledort *et al.*, 1994; Aznar *et al.*, 2000; Jayandharan & Srivastava, 2008; van Dijk *et al.*, 2005a). The clinical phenotype of severe haemophilia consists of spontaneous joint and muscle bleeds. These frequent joint bleeds will lead to irreversible arthropathy (Bolton-Maggs & Pasi, 2003; Jayandharan & Srivastava, 2008). About 10-15% of patients with FVIII levels of < 1.0 IU/dL have clinically mild disease in terms of frequency of bleeds, extent of arthropathy and lower requirement for factor infusion (Janco *et al.*, 1996). The age at which the individuals with haemophilia experience their first joint bleeds also varies considerably. Most individuals with severe haemophilia experience their first bleed by age 3-5 years, however, it has been reported that some individuals can bleed before the age of one year (Fischer *et al.*, 2002). It is also known that patients from the same family, carrying the same gene defect, may not have an identical bleeding tendency (Barnes *et al.*, 2007).

The mechanism of this phenotypic heterogeneity is not clearly understood (Dargaud *et al.*, 2004). Many environmental and genetic differences between patients could account for this phenotypic heterogeneity. Recent studies have suggested that the presence of FV_{Leiden} (FVL) G1691A can modify the clinical phenotype in haemophilia (Franchini & Lippi, 2009; van Dijk *et al.*, 2004). Resistance to the anticoagulant effect of activated protein C (APC) is the most frequent inherited risk factor for venous thromboembolism (VTE) (Dahlback, 1994). The Leiden mutation in exon 10 of the

factor V gene is responsible for the majority of cases that have prevalence of 2-7% among Caucasian populations. Heterozygous carriers of FVL have approximately 5-fold increased risk of VTE, whereas homozygous carriers have a 20-80-fold increased risk (Lee *et al.*, 2000).

The prothrombin G20210A mutation is another inherited risk factor for VTE. Prothrombin is the circulating precursor of thrombin, which plays a central role in haemostasis, in particular, the formation of fibrin. A mutation in the 3'-untranslated region of the prothrombin (PT3') has been associated with elevated plasma prothrombin levels which may increase the risk of VTE (Tizzano *et al.*, 2002b).

AT, PC, and PS deficiencies are well established risk factors of VTE and the coexistence of any or combined deficiencies of these proteins has been reviewed or studied by many investigators (Escuriola Ettingshausen *et al.*, 2001; Nowak-Gottl *et al.*, 2003). Increased levels of other procoagulant factors such as FVIII, FIX, and VWF (van Dijk *et al.*, 2004), or decreased levels of FXII (Johnson *et al.*, 2010) have also been shown to confer an increased risk of thrombosis and are also candidate modifiers of the haemophilia phenotype.

The underlying mutation in *F8* or *F9* may also play a role in clinical phenotype expressions. The type of gene mutation is responsible for the residual FVIII or FIX levels in the plasma, therefore, the molecular defects that totally prevents the synthesis of the protein (null mutation) are usually associated with undetectable factor activity and antigen, whereas non-null mutations account for variable factor levels.

6.2. Aims

In this chapter, the aims of the study were:

- To identify the presence of concurrent pro-thrombotic or anti-thrombotic factors that may contribute to the heterogeneity of thrombin generation in haemophilia patients.
- To investigate the influence of the factor VIII gene defect among the severe HA individual on the clinical phenotype.
- To investigate the possibility of certain clinical features to predict the severity of the disease. The markers chosen for correlation included:
 - age of first bleed,
 - The number of joint bleeds per year,
 - The amount of treatment (factor concentrate) consumption per year may indicate the severity.

6.3. Study subjects

Ninety seven haemophilia patients were enrolled in the study, of which 73 included after excluding those who had residual FVIII/FIX (see section 2.1.2). These comprised 36 severe, eight moderate, 15 mild haemophilia A, and seven severe, three moderate, and four mild haemophilia B, aged between 18 and 90 years (mean 43, median 41 years). Patients on prophylaxis or on demand treatment were asked to omit treatment for a minimum of 72 h to allow the FVIII or FIX level to return to baseline level. Those who required treatment were excluded from study. The study was approved by the ethics committee of the Royal Free Hospital, and informed consent was obtained from patients and healthy individuals. The normal reference ranges

(Table 6.1) were taken from the Katharine Dormandy Haemophilia Centre and Thrombosis Unit (KDHCTU) Laboratory.

6.4. Results

6.4.1. Prothrombotic risk factors

Pro-thrombotic risk factors including FVL, PT3', PC, PS, and AT were investigated by performing the assays as described in section 2.4. Table 6.1 summarises the results.

Table 6.1 Prothrombotic risk factors in haemophilia

	FVL (Absent)	PT3' (Absent)	AT (81-115 IU/dL)	PC (58-137 IU/dL)	Free PS (67-127 IU/dL)	VWF:Ag (45-171 IU/dL)
Severe HA <i>n</i> = 36	2 Heterozygous	1 Heterozygous	None	1	1	5
Moderate HA <i>n</i> = 8	None	None	2	1	2	3
Mild HA <i>n</i> = 15	None	1 Heterozygous	1	1	None	3
Severe HB <i>n</i> = 7	None	None	None	None	None	2
Moderate HB <i>n</i> = 3	None	None	1	None	None	None
Mild HB <i>n</i> = 4	None	None	1	1	None	1

Prothrombotic risk factors (reference range IU/dL in brackets) investigated in all patient groups. The number of effected individuals for each risk factor has been illustrated in this table. HA = haemophilia A, HB = haemophilia B, FVL = factor V Leiden, PT3' = prothrombin 3' untranslated region mutation, AT = antithrombin, PC = protein C, Free PS = free protein S, VWF:Ag = von Willebrand factor antigen.

Abnormal AT, PC, and free PS levels for all patient groups ranged 65-78 IU/dL, 40-68 IU/dL, and 44-65 IU/dL respectively. These abnormalities were thought to be due to the chronic liver disease as a result of hepatitis C. Liver function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ -glutamyl transferase (γ -GT) were performed on all samples to exclude those with hepatic failure from the study as outlined in the exclusion criteria (section 2.1.2). However, it

was assumed that some individuals with normal liver function tests may still exhibit some degree of hepatic abnormalities through their abnormal AT, PC, and free PS. After discussion with the Haemophilia Consultant Haematologist, the abnormalities of AT, PC, and free PS were not considered as sufficiently clinically significant to affect the thrombin generation results.

VWF:Ag levels were increased in 14 individuals (range 172-300 IU/dL, reference range 45-171 IU/dL). The increased VWF:Ag was also believed to be related to the liver abnormalities and/or HIV infection as result of the inflammatory response (as discussed with the Haemophilia Consultant Haematologist). However, increased VWF:Ag levels are known to be a thrombotic risk factor which may influence the bleeding phenotype of individuals. The severe HA group was divided into two subgroups of normal VWF:Ag and increased VWF:Ag levels. The Mann-Whitney *U* statistical test showed no significant difference between PH+CTI or AUC+CTI of two subgroups ($p = 0.157$, and 0.314 respectively).

6.4.2. Procoagulant factors

Other coagulation factor levels were also analysed to investigate any co-existent deficiency of procoagulant factors, including FII, FV, FVII, FX, and FXI (section 2.4). Table 6.2 illustrates the number of individuals with decreased procoagulant factors.

The majority of individuals with decreased FXI activity levels ($n = 11$, FXI activity levels between 55-69 IU/dL) were reviewed by the Haemophilia Consultant Haematologist and the abnormality were considered not to be clinically significant, and not to cause any adverse effect on their bleeding phenotype. One individual,

however, was considered to have a clinically significant low level of FXI (FXI = 36 IU/dL) and further genetic analysis was requested to confirm if genetic mutation existed within the FXI gene (result of mutational analysis was not available at time of presentation of this thesis).

The Mann-Whitney *U* statistical test showed no significant difference between PH+CTI or AUC+CTI parameters of TGT in severe HA with decreased FXI levels and those with normal FXI (*p* = 0.350, and 0.306 respectively).

Table 6.2 Procoagulant factors

	FII (72-128 IU/dL)	FV (50-127 IU/dL)	FVII (61-150 IU/dL)	FX (64-125 IU/dL)	FXI (70-145 IU/dL)
Severe HA <i>n</i> = 36	1	1	None	1	12
Moderate HA <i>n</i> = 8	None	None	None	None	4
Mild HA <i>n</i> = 15	None	None	None	None	2
Severe HB <i>n</i> = 7	None	None	1	None	2
Moderate HB <i>n</i> = 3	None	None	None	None	None
Mild HB <i>n</i> = 4	None	None	1	None	None

Co-existence of other procoagulant factors investigated in all patient groups (reference range in brackets). The number of affected individuals for each risk factor has been illustrated in this table. HA = haemophilia A, HB = haemophilia B.

The number of individuals with FVL or PT3' mutation, low natural inhibitors or prothrombotic factors was not statistically significant to perform any comparative statistical analysis. Descriptive statistical analysis was performed to compare the most sensitive parameters of ROTEM[®], TGT, CAT, and clot waveform analysis.

One severe HA individual with heterozygous FVL had increased PH (35%) in PRP+CTI category of the in-house TGT and shorter ttPeak (13.5 min) of the CAT when compared to the relevant median (29% and 16 min respectively), whereas another individual with the same mutation did not show the same trend. There was one severe HA individual with the PT3' mutation and borderline PS level of 63 IU/dL (reference range 67-127 IU/dL) who had higher Peak (18%) and ETP (50%) than the median Peak (16%) and ETP (31%) in severe HA group. One severe HA individual, with FXI level of 36 IU/dL, had reduced Peak (5%) and ETP (15%), and longer TP (38 min) than the relevant median (16%, 31%, and 20.5 min respectively).

6.4.3. Mutational analysis

The mutational data was obtained from the Genetic Laboratory of KDHC&TU. Of 36 severe HA subjects, 32 mutational analyses were available to date. Nineteen individuals had intron 22 inversion (59%), of which 12 were distal (63%) and 7 were proximal (37%). There were 7 missense mutations (23%), three large deletions (> 200bp deletion) (9%), two insertion (6%), and one small deletion (< 200bp deletion) (3%). The mutational analysis of the severe HA group was divided into two subgroups: null mutations and non-null mutations. The null mutations consisted of inversions ($n = 19$) and large deletions ($n = 3$); and non-null mutations consisted of missense mutations ($n = 7$), small deletion ($n = 1$), and insertions ($n = 2$). FVIII antigen analysis was performed on all samples and individuals with FVIII antigen above 1.0 IU/dL were excluded from the null mutation subgroup.

The two groups were compared by TGT, CAT, ROTEM[®], and clot waveform analysis parameters. The Mann-Whitney *U* test showed significant differences between individuals with null mutations and non-null mutations subgroups by PPP+CTI AUC% ($p = 0.0014$) (Figure 6.1), PPP+CTI PH% ($p = 0.0076$) (Figure 6.2) parameters of the TGT; PPP+CTI ETP% ($p = 0.0084$) (Figure 6.3), PPP+CTI Peak% ($p = 0.0096$) (Figure 6.4) parameters of the CAT; and CWB+CTI+TF tMaxVel ($p = 0.0455$) parameter of the ROTEM[®]. However, considering the sensitivity and specificity of these parameters in hypocoagulability, the PH in PPP+CTI test category with both sensitivity and specificity of 94% would be the best test in differentiating between the two subgroups of null and non-null mutation severe HA.

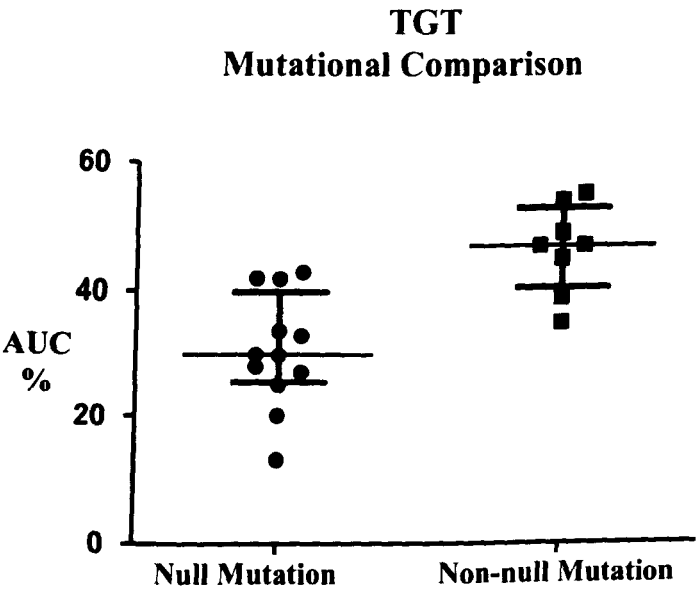


Figure 6.1 TGT Mutational comparison (AUC)
The AUC% compared between null Mutations and non-null Mutations subgroups of severe HA. Significant differences were found between PPP+CTI AUC% parameter of TGT ($p = 0.0014$).

TGT
Mutational Comparison

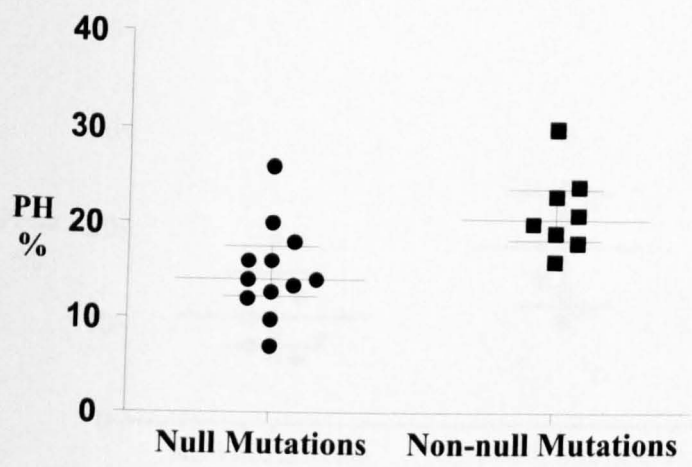


Figure 6.2 TGT Mutational comparison (PH)
The PH% compared between null Mutations and non-null Mutations subgroups of severe HA. Significant differences were found between PPP+CTI PH% parameter of TGT ($p = 0.0076$).

Thrombogram
Mutational comparison

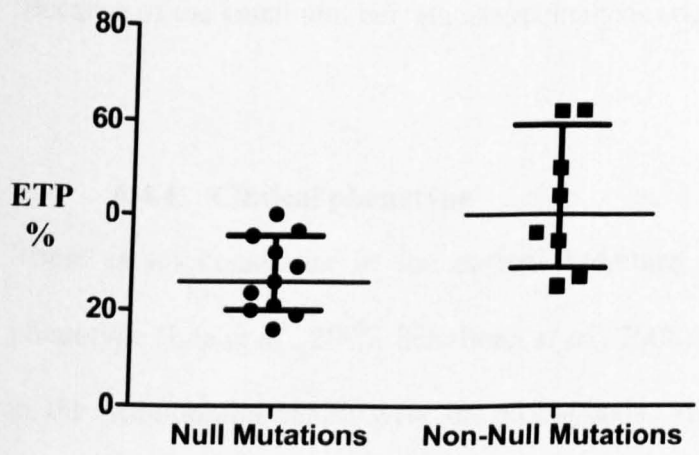


Figure 6.3 CAT mutational comparison (ETP)
The ETP% compared between null Mutations and non-null Mutations subgroups of severe HA. Significant differences were found between PPP+CTI ETP% parameter of the CAT ($p = 0.0084$).

Thrombogram Mutational comparison

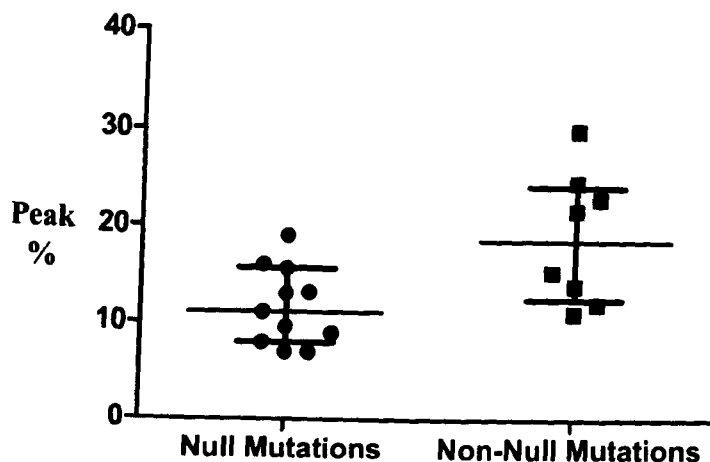


Figure 6.4 CAT mutational comparison (Peak)

The Peak% compared between null mutations and non-null mutations subgroups of severe HA. Significant differences were found between PPP+CTI Peak% parameter of the CAT ($p = 0.0096$).

In the severe HB group there were seven individuals with FIX levels of < 1 IU/dL, of which five individuals had missense mutations and two had deletions in their *F9*.

Because of the small number, statistical analysis could not be performed.

6.4.4. Clinical phenotype

There is no consensus in the current literature on how best to define clinical phenotype (Lee *et al.*, 2005; Schulman *et al.*, 2008), furthermore most of the patients in the haemophilia centre were on prophylaxis. The patients on demand treatment have been offered prophylaxis in the past and have refused, and therefore is a self selected group of non severe phenotype. A clinical discussion forum was set up with two medical consultants (Dr Pratima Chowdary, Consultant Haematologist, and Dr

Thyn Thyn Yee Associate Specialist) who knew the patients' history in view of long term contact and three specialist nurses (Patricia Lilley, Barbara Subel, and Debra Pollard) who knew the patients for more than 10 years. Following active discussion and debate, it was agreed that the following data be extracted from the medical notes and be correlated independently to the global coagulation measures:

- The age of first joint bleed.
- The average usage of treatment during the last five years (factor replacement concentrate per kg of the individual's body weight, per year). This relates to the use of prophylaxis, and in patients on an on-demand treatment regimen it relates to the number of bleeds.
- The number of joint bleeds per year (the average of the last five years).
- Treatment regimen - regular prophylaxis versus on demand treatment. All severe patients have been offered prophylaxis, and most patients who have a significant bleeding tendency tend to continue prophylaxis. Patients with not so severe bleeding tendency tend to either be on prophylaxis or switch to on-demand treatment based on their personal circumstances and choice.
- Clinical impression of bleeding tendency - there is a very slow turnover of patients and staff in the centre, and it was felt that a clinical impression might provide a valuable clue on the bleeding tendency. A list of participants was given to two consultants and three senior nurses who had been involved in treating and caring for the participants throughout their registration with the KDHCTU.

The Spearman correlation statistical analysis was performed to investigate if there was any correlation between the laboratory test parameters and clinical phenotype parameters.

The age at the first joint bleed correlated significantly with all three parameters in-house TGT in PRP+CTI category (Table 6.3). The ETP parameter of the CAT and time to peak (TP) of PPP+CTI category of the in-house TGT were also found to correlate with the age at the first joint bleed ($r = 0.426$, $p = 0.043$; and $r = -0.432$, $p = 0.028$, respectively).

Table 6.3 Clinical phenotype correlation with TGT, age at the first joint bleed.

Age at the first bleed ($n = 23$)	r	p
PRP+CTI AUC%	0.693	0.003
PRP+CTI PH%	0.718	0.002
PRP+CTI TP	-0.703	0.002
ETP	0.426	0.043
PPP+CTI TP	-0.432	0.028

PRP TGT appeared to be strongly correlating with the age at the first joint bleed. The ETP and TP in PPP+CTI test category also weakly correlated with age at the first joint bleed. TGT = thrombin generation test, PRP = platelet rich plasma, PPP = platelet poor plasma, CTI = corn trypsin inhibitor, AUC = area under the thrombin generation curve in in-house TGT, PH = peak height thrombin generation in in-house TGT, TP = time to peak thrombin generation in in-house TGT, ETP = endogenous thrombin potential in the calibrated automated thrombogram*.

The number of joint bleeds per year was significantly correlated with the ETP parameter of the CAT ($r = -0.601$, $p = 0.014$). The factor concentrate consumption

(IU/Kg/year) correlated significantly with the Min1 ($r = 0.647$, $p = 0.001$) and Min2 ($r = 0.526$, $p = 0.036$) of the clot waveform analysis.

The Mann-Whitney U test showed significant difference between the PH PPP+CTI category of the TGT of those individuals who were on prophylaxis and those who were on demand treatment regimens ($p = 0.0047$). The median PH in the on prophylaxis group ($n = 21$) was 14% (inter-quartile range: 12-20%) whereas the median PH in the on demand group ($n = 14$) was 20% (inter-quartile range: 16-29%). This result showed that the on-demand group of severe HA patients may have less severe bleeding phenotype than those on prophylactic regimen.

6.5. Discussion

A large heterogeneity in thrombin generation was noticed in patients with severe HA and HB using different global assays, as described in previous chapters. In this chapter, the aim was to investigate the existence of modifying factors that may contribute to this heterogeneity between individuals with severe HA or HB. Prothrombotic, and procoagulant factors were analysed and their effects were investigated on thrombin generation, ROTEM[®], and clot waveform analysis.

There were two individuals that were heterozygous for FVL in the severe HA group, accounting for 5.7% of the total severe HA group, which is consistent with the prevalence of FVL in Europe (Gray *et al.*, 2006). The PH+CTI parameter of the TGT was increased and the time to peak parameter of the CAT was shorter in one subject. There was also one severe HA patient who was heterozygous for PT3' and whose

thrombin generation showed an increase in PH+CTI and ETP levels, compared to the median the same group. The number of affected individuals was not enough to perform statistical analysis, and prove or disprove whether presence of a prothrombotic risk factor may have influenced the PH and ETP. However, the results were in agreement with an earlier report that no association was found in the cohort patients to confirm or reject the modulation of bleeding phenotype in the presence of a prothrombotic risk factor (Beltran-Miranda *et al.*, 2005).

The co-existence of other mild inherited bleeding disorders was also investigated and one individual with low (clinically significant) FXI level was identified. Peak height TGT was reduced and time to peak was increased in this individual, reflecting a possible adverse effect of low FXI level on thrombin generation. Clinically, the individual is one of the severe bleeders. His first bleed was at the age of one month, and started prophylaxis regimen at the very early age. He had one major joint surgery.

The type of mutation was significantly related to an individual's thrombin generation capacity. The null mutation subgroup showed a significantly reduced thrombin generation when compared to the non-Null mutation subgroup. This was shown by AUC ($p = 0.0014$), PH ($p = 0.0076$), ETP ($p = 0.0084$), and Peak ($p = 0.0096$) thrombin generation methods in PPP+CTI test category, and also time to maximum velocity ($p = 0.0455$) of ROTEM[®] in CWB+CTI+TF test category. A recent study also showed that ETP was significantly higher in individuals with less severe types of *F8* mutations among severe HA patients (Santagostino *et al.*, 2010).

The most common predictors of the clinical phenotype of severe HA patients included age at first joint bleed, bleeding frequency, factor consumption and orthopaedic scores (Aledort *et al.*, 1994). However, since the introduction of primary prophylaxis to prevent bleeding episodes, the natural history of the severe haemophilia has changed dramatically, and the clinical phenotype is more difficult to define (Santagostino *et al.*, 2010). In the multidisciplinary discussion held in the Haemophilia Centre, attended by two consultant haematologists and three specialist nurses, there was agreement that amongst adult patients, an on demand regimen was generally preferred by patients who have a less severe bleeding tendency. Patients on prophylaxis, as a group may either have a known previous severe bleeding tendency, severe joint damage or are disciplined to receive regular prophylaxis.

The total factor consumption was felt to be a reasonable surrogate marker of the bleeding tendency, as was demonstrated by Schulman *et al.*, (2008). Factor consumption per year was documented for 5 years, and the average IU/kg/year was calculated for correlation. As the patients enrolled in the study included patients on primary and secondary prophylaxis, it was felt that the joint scores may not truly represent the underlying bleeding tendency for correlation.

The study described in this thesis showed that the PH parameter of the in-house TGT was significantly higher in the severe HA on-demand group than those who were on a prophylaxis regimen. However, other parameters of thrombin generation, ROTEM^{*}, or clot waveform analysis did not show a significant difference between the two groups. Several studies have confirmed that age of first joint bleed is variable in patients with severe haemophilia (Bolton-Maggs & Pasi, 2003; Jayandharan &

Srivastava, 2008; van Dijk *et al.*, 2005a). In a recent study, Santagostino *et al.* (2010) reported an association between the severity of clinical phenotype of severe haemophilia and ETP measured in PRP. The authors reported that no correlation was found between the severity of clinical phenotype and thrombin generation in PPP, for which they referred to the role of platelets in thrombin generation. Similarly in the study described in this thesis, AUC, PH, and TP parameters of in-house thrombin generation measured in PRP+CTI strongly correlated with the age at first joint bleed. In addition, the ETP parameter of the CAT, and the TP parameter of in-house TGT measured in PPP+CTI were also correlated, to a lesser extent, with the age at the first joint bleed.

The involvement of platelets in thrombin generation reflects *in vivo* conditions as the activation of platelets is crucial for initiation and propagation phases of thrombin formation. In a short report Dargaud *et al.* (2006) compared ETPs of two severe HA patients with five healthy controls to examine the function of platelets. They observed that when contact factor activation was eliminated, the thrombin-platelet feedback loop was a major determinant of thrombin generating capacity and platelets had a greater role in regulating the propagation of thrombin generation than its initiation. In the study described in this thesis, CTI was used to eliminate the contact activation, which may have enhanced the sensitivity of the assay compared to study by Santagostino *et al.* (2010).

Interestingly, only parameters of clot waveform analysis correlated significantly with the factor concentrate consumption (Min1, $r = 0.647$, $p = 0.001$ and Min2, $r = 0.526$,

$p = 0.036$). No correlation was found between factor consumption and TGT or ROTEM[®] parameters.

6.6. Conclusion

Although the results suggest that clinical phenotype can be predicted by some parameters of thrombin generation and thromboelastometry, the prophylaxis regimen does alter the bleeding tendency and indeed more than 50% of the individuals in this study were on a prophylactic regimen. However, among the clinical phenotype predictors, age at first bleed is an important marker as prophylaxis is yet to start. The TGT in platelet rich plasma, with contact factor inhibition, was shown to be the most sensitive parameter for predicting the clinical phenotype of individual patients.

Chapter 7 **General discussion and conclusion**

7.1. General discussion

The ability to predict severe bleeding or thrombotic tendency is an important goal for patients and their treating clinicians for individualised management enabling cost effective treatment. This goal has remained largely elusive because individual molecular markers of coagulation do not provide an overall picture of an individual's haemostatic balance. In the study of haemostasis, and the investigation of patients for bleeding or thrombotic conditions, there is a renewed interest in assays that evaluate net clotting potential, or the generation of key enzymes in the coagulation system (e.g., thrombin), which provide a comprehensive fingerprint of a patient's haemostatic state. In this study, the use of three different global assays was evaluated and the potential for use in regular clinical practice in patients with haemophilia was investigated.

All the three global assays, ROTEM[®], TGT, and clot waveform analysis, were shown to be reliable diagnostic tests to identify individuals with lower than normal coagulation FVIII or FIX levels. The heterogeneity of thrombin generation, in particular in severe haemophilia A and B, was also demonstrated by all three global assays. This indicates that the rate and amount of thrombin generation are not exclusively dependent on the amount of FVIII or IX level in the samples.

The use of CTI to eliminate the interference of the contact system was also shown to play an important role in improving the accuracy and precision of thrombin generation. The PH in PPP+CTI, and PH and TP in PRP+CTI in-house TGT were the

most sensitive and specific parameters to distinguish between thrombin generation potential of normal and haemophilia patients. This confirms earlier reports by other groups that the use of CTI at low TF concentration (1.0 pM) for initiation of thrombin generation improves assay variations (Dargaud *et al.*, 2005; Luddington & Baglin, 2004; van Veen *et al.*, 2008)

The correlation between the results of in-house TGT and CAT method was not strongly significant despite the fact that the methods are similar in principle. Such discrepancies are not uncommon, as shown in recent multicentre studies where the use of different tissue factor and phospholipid sources was shown to produce a large variability in thrombin generation results (Dargaud *et al.*, 2009; Gerotziafas *et al.*, 2005). In the study in this thesis, the tissue factor source and the dilution factor for the in-house method was similar to the CAT starting reagent, but the CAT reagent was supplied in a lyophilised form in which the phospholipids are incorporated in to the reagent. For the in-house method the tissue factor was freshly made and diluted just prior to testing, followed by addition of freshly made phospholipids to the plasma sample. This may explain the precision and accuracy of the in-house TGT demonstrated by ROC analysis in which the in-house TGT produced more sensitive and specific results than the CAT assay. A large heterogeneity in severe HA and HB groups was demonstrated by the three global assays which were employed in this study. This could be explained, in part, by the fact, that the clearance of FVIII/FIX prophylaxis treatment is different between individuals. The effort made to ensure no residual factor concentrate remained after appropriate hours off treatment, by the exclusion of 13 HA patients who had FVIII activity levels of > 1.0 IU/dL, explains the heterogeneity in clearance of residual treatment. In a study by van Dijk *et al.*

(2005), it was shown that severe HA, treated prophylactically with factor concentrates, those with a shorter FVIII half-life required slightly more clotting factor to prevent joint bleeds and subsequent arthropathy than similar patients with a longer FVIII half-life. Therefore it would be more appropriate to conduct such studies before the start of prophylactic regimen i.e. newly diagnosed children or adults. On the other hand, however, this study showed that the type of mutation in severe HA is significant in thrombin generation potential. It has been speculated that patients with “severe molecular defects” (inversions, large deletions and nonsense mutations) produce no FVIII, whereas those with “less severe molecular defects” may produce a small amount of circulating FVIII (i.e. FVIII antigen), but their FVIII clotting activity is remain less than 1.0 IU/dL as measured by one-stage clotting factor assay (Oldenburg, 2001; Schwaab *et al.*, 1995).

Clot waveform analysis was carried out in this study, and showed that heterogeneity exists among the severe haemophilia A and B individuals, and was more pronounced in Min1 and Min2 parameters when compared to the APTT. Min1 and Min2 appear to be sensitive, simple, fast and cost effective in diagnosing hypocoagulability and may be useful in the monitoring of haemophilia patients.

The coefficient of determination (r^2) for Min1 and FVIII was 0.624 (Figure 5.3 chapter 5), suggesting that more than 62% of variance in Min1 axis can be explained by variance of FVIII axis. The r^2 for Min2 was 0.568 which also shows that 57% of the variance in the Min2 axis can be explained by variance of FVIII activity levels axis. However, the APTT was shown to be a weak predictor to explain the variance of FVIII with r^2 of 0.229. This may indicate that the time to clot formation may be

affected by other modifying or interfering factors, whereas such influence on the rate and the acceleration of clot formation may be less effectual than the deficient factor.

The analysis of clinical phenotype has proven to be difficult and other investigators have also experienced similar problems. There are conflicting observations by different groups on correlation of global assays with the clinical phenotype of haemophilia patients. While some have found parameters of thrombin generation to correlate with the clinical phenotype (Dargaud *et al.*, 2005; Santagostino *et al.*, 2010), others have not found such a correlation (Beltran-Miranda *et al.*, 2005). However, this study identified that thrombin generation in platelet rich plasma correlated more significantly than platelet poor plasma or the whole blood, with age of first joint bleed, a clinical marker most often used in scores for defining the clinical phenotype.

It has been well established that platelets play a central and crucial role in generation of thrombin both in the primary and the secondary phases of haemostasis. Several studies have shown the inter-individual differences in platelet function and concluded that this phenomenon may play a part in modifying the clinical phenotype of severe haemophilia (Dargaud *et al.*, 2005; Monroe *et al.*, 2002b; Santagostino *et al.*, 2010; Siegemund *et al.*, 2003; Yee, 2006). In the study described in this thesis, significant correlation was found between the age at first joint bleed of the severe HA group and PH, AUC, and TP parameters of the in-house TGT in PRP+CTI category. The age of first joint bleed is thought to be one of the most reliable indicators to differentiating between the severe patients and non severe patients. (Escuriola Ettingshausen *et al.*, 2001; Van den Berg *et al.*, 2007). Although, it should be acknowledged that due to limitations, we could not obtain clinical data for all participants in this study and only 23 individuals were entered in this analysis, therefore the significance level was

reduced to $p < 0.01$ to reject the null hypothesis. However, the level of significance (p) for this correlation was 0.002, 0.003, and 0.002 for PH, AUC, and TP respectively, which indicates that correlation is not just by coincidence.

It is well established from clinical practice that those individuals who are on an on-demand treatment regimen have less bleeding frequency than those who are on regular prophylaxis. Subgroups of 9-10% of patients with severe HA, showing only little radiological joint damage while treated on-demand have been described both by Aledort *et al.* (1994) and Molho *et al.* (2000). Therefore, theoretically, the thrombin generation potential of the former group should be higher than the latter group. This study showed that the PH parameter of in-house TGT (PPP+CTI) was significantly higher ($p = 0.0047$) in the severe HA on-demand group than those who were in the on prophylactic regimen group. However, other parameters of TGT or other assays did not show a similar trend.

The type of *F8/F9* mutation is also known to predict the clinical phenotype. Severe defects such as intron 22 of *F8* in severe HA patients are always associated with a severe clinical phenotype, whereas missense mutations are known to cause a less severe clinical phenotype (Jayandharan *et al.*, 2005). However, in this study the heterogeneity of results were noted in all parameters of global assays. Despite this heterogeneity, the result of in-house TGT, CAT, and to a lesser extent ROTEM[®] showed a significant difference between the null and non-null mutations subgroups.

The global assays evaluated in this study assessed coagulation, but the fibrinolytic pathways were not addressed, which are clinically relevant. It was not in the scope of

this study to investigate the fibrinolysis pathway of haemostasis in our group of individuals, as most current assays for assessing fibrinolysis are not global assays, and are most helpful in identifying defects of the fibrinolytic pathway, but are unlikely to assess the impact of low thrombin generation on the overall fibrinolysis. There are assays which assess thrombin activatable fibrinolysis inhibitor (TAFI) activity, however, to date, only one study has suggested an association of the fibrinolytic activity and the bleeding phenotype in haemophilia A and B individuals (Grünewald *et al.*, 2002). In their study, Grünewald *et al.* showed that the median tissue-type plasminogen activator (t-PA) was significantly elevated in patients with a more severe phenotype. They hypothesised, that the higher frequency and/or intensity of bleeding events in the severe bleeder group (compared to a less severe bleeder group) of their study induced the generation of more fibrin through repeated or prolonged stimulation of procoagulant mechanisms. Increased availability of fibrin in turn facilitated the activation of the fibrinolytic system.

Inter-individual variations in the pharmacokinetics of FVIII may also play a part in the bleeding phenotype. Thus variability of FVIII/FIX clearance may have an impact on clinical phenotype in patients on regular prophylaxis, as suggested previously (Escuriola Ettingshausen *et al.*, 2001; van Dijk *et al.*, 2005b). Taking into account the limitations of one-stage clotting assay at FVIII/FIX level of < 1.0 IU/dL, it is difficult to exclude the presence of residual treatment material, which could have an impact on the global assays, thus undermining some of the negative correlations in this study.

7.2. Limitations of the study

In this study although 49 severe HA and 14 severe HB were consented. Due to slow clearance of FVIII/FIX, detectable levels were found in the plasma of 13 severe HA and seven severe HB, and consequently excluded from further data analysis. In particular in severe HB patients, 5 days washout period proved to be inadequate for clearance of the residual FIX. This caused a limitation in interpretation of global assays in the severe HB group. The small number of participants in moderate and mild HB groups also limited the interpretation of results, primarily because of the closeness of their median FIX activity levels (median FIX in moderate HB = 2.0 IU/dL, mild HB = 5.0 IU/dL). This may have caused a bias in differentiation power of TGT, ROTEM[®], and clot waveform parameters to distinguish between moderate and mild HB groups. Therefore, it is suggested that for future studies for identification of the baseline levels, due consideration is given to the individual pharmacokinetic profile before recommending the washout period.

7.3. Conclusion

The use of global assays in demonstrating the coagulation potential of haemophilia patients proved to be useful, sensitive and specific compared to conventional methods, which assess individual factor levels. However, the complex nature of global assays, make them difficult to interpret on their own. Therefore, these assays should be used in parallel with the conventional tests in order to be more useful. The lack of standardisation for these assay does limit their use in routine clinical practise as reproducibility of laboratory assays is one of the main principles of their validity,

which should be comparable not only within the laboratory but also between laboratories at local, national and international level.

For correlation with clinical phenotype, careful attention to the preanalytical variables is important and efforts should be made to eliminate as many confounding factors as possible in order to obtain a bias free outcome. The use of CTI proved to make TGT and ROTEM[®] parameters more sensitive. Although, in correlating clinical phenotype with parameters of global assays, some of the confounding factors cannot be reversed such as the effect of prophylaxis on clinical phenotype.

7.4. The use of global assays in local routine practice

The outcome of this study has generated a huge interest among the KDHCTU clinicians, scientists, and nurses. Particularly, those who were involved in the care and recruitment of individuals participated in the study. The KDHCTU treats and cares for a number of haemophilia patients who have developed an inhibitor toward FVIII/FIX, and also a number of acquired haemophiliacs who are registered at the KDHCTU. Monitoring these patients with conventional assays is not optimal and the use of global assays has already been proved useful by other investigators (Ingerslev *et al.*, 2000; Sørensen & Ingerslev, 2004a). However, the use of CTI to minimise the preanalytical variables in ROTEM[®] analysis was novel in this study, and since the outcome, the practice has been changed. The use of clot waveform analysis has also being considered in a group of HB patients who have undergone gene therapy in the KDHCTU, and once analysed, it will be published.

The publication of outcome will be the next step, as the interest in this field, particularly the use of three different global assays, is growing. Although two posters have already been presented in two international events on thrombosis and haemostasis, detailed publications in form of scientific paper are necessary to inform the interested researchers in this field about the outcomes of this study.

7.5. Future studies

Global assays are undergoing a revival since it is increasingly recognised that conventional single factor assays do not always predict the clinical phenotype either in patients with inherited bleeding disorders or in patients with thrombotic conditions. Furthermore these assays, to varying extents, represent the complex interactions between the various coagulation factors and cellular components. In clinical laboratories there is a great need for such tools to be able to characterise patients with increased risk of thrombosis or bleeding, and for optimisation of anti-thrombotic or anti-bleeding therapy.

For global assays to be clinically relevant validation of the tests is required by correlation with clinical outcomes. Indeed the TGT, CAT, and some ROTEM^(R) parameters have shown a significant correlation to the clinical markers. Unfortunately due to the limitations eluded to earlier, the clinical correlation has been less than satisfactory. Therefore, further studies are required with more robust clinical endpoints to better define the role of these assays in the routine clinical care of

patients. This is particularly relevant as these assays which identify the heterogeneity of coagulation in these disorders are ideally suited for individualised treatment of patients once appropriate thresholds for clinical outcomes are defined.

Due consideration needs to be given to the role of preanalytical variables including the type of anticoagulant used. A recent study has shown that the calcium chelation may affect dynamics of TF initiated thrombin generation as well as the whole blood thromboelastometry (Mann *et al.*, 2007). Moreover, it has been documented that citrate and calcium chelation interferes with the metabolic processes in platelets (Schneider *et al.*, 1997). Therefore CTI as an alternative anticoagulant may be employed in a future study to reflect more accurately the *in vivo* scenario.

Chapter 8 **Reflection**

“Tell me and I forget, show me and I remember, involve me and I understand”

(Confucius about 450 B.C.)

The purpose of this chapter is to reflect upon my experience during the completion of the Professional Doctorate in the Biomedical Science (DBMS). By expressing my feelings about the events, I could understand my weaknesses and find ways for improvement. (Brookfield, 1986) suggests that educational programs should be interactive, include reflective components, and be related to experience. By including these components, learners are given more autonomy and are encouraged to take charge of their own learning. My aim is to provide an insight into how to use the "self-reflection" (Schön, 1983) and "writing" (Greenwood, 1991) processes as two important means of personal and professional growth.

8.1. The need for continuous professional development

Despite having an MSc degree in the field of haematology, I felt that I needed to develop my professional expertise in this field to an advanced level with high quality academic knowledge to support my practice. On the other hand, the Institute of Biomedical Science (IBMS) continuous professional development (CPD) programme to maintain, improve and broaden the professional knowledge and skills was another motivating factor to search for a way to continue my personal development in a structured approach.

Having read about the DBMS course at the University of Portsmouth, I felt that this was the right course to embark on in order to fulfil my learning needs. I started the DBMS in October 2003 to accomplish my learning needs.

The first part (Stage 2, Part 1) of the course was interesting, challenging and motivating. The importance of reflective practice, as part of the learning process, was emphasised. I learnt that it is impossible to memorise every event and experiment in my mind for a later date and to return and learn from them or try to remember what happened and learn from it. Then the reflective writing was introduced and I learnt to write the events down so that I can pull them together into a coherent body of knowledge and come to recognise the totality of what I know. There is something in writing that enables us to recapture the experience and reflect on it and learn from it. Thus, in reflective practice the purpose of reflective writing is learning (Jasper, 2005).

The Advanced Research Techniques Unit was very specific to the needs of the individual to understand the process of conducting a research project. The preparation of a resource pack for a new diagnostic test before its introduction into the laboratory was a unique experience. By writing the resource pack, I learnt how to apply the research into practice, and what steps needed to be taken to facilitate this. The unit helped me to design the research project and follow the steps to:

- identify the appropriate research question
- select the appropriate study design
- prepare a research protocol
- obtain an ethics committee approval
- sample the research population in accordance with accepted criteria

- conduct the study in a planned, structured approach
- analyse and interpret the findings using the appropriate statistical tests
- report and disseminate findings

The critical appraisal of literature was a valuable experience and I gained a good deal of confidence from it. It gave me skills that allowed me to make sense of research evidence, to assess the quality of the research, and to determine the best evidence for practice. It was a great help to me in the second part of the course, “*the Professional Research and Development Project*”, in order to appraise, evaluate and compare different methodologies and studies in preparation of the Professional Research and Development Proposal. As Sackett (1996) indicated, evidence-based medicine is the conscientious, explicit and judicious use of current best evidence in making decisions about the care of individual patients. Boud *et al.* (1985) also state that to maximise learning through critical reflection we need to locate ourselves within the experience and explore the available theory and knowledge to understand the experience in different ways.

The peer review process, as a quality control for draft articles, involved the critical appraisal of a draft article for publication in a chosen journal with reference to the *instruction to authors* of that particular publisher. In this process I learned how to examine the benefits and shortcomings of a research study, and to recommend improvements or request reanalysis of data before publication. The peer review process allows the reader to make some judgment about the relative quality and merit of the research. It stops a lot of substandard and poor science from reaching publication.

However, the process of peer review is not perfect. The manuscript may be judged on the reputation of the author instead of quality. An excellent paper written by a new scientist can be rejected, whilst a poorer but uncontroversial paper by an established researcher can sail through the peer review process. The reviewers or referees may vary markedly in their opinions. Reviewers may use their anonymity as justification for being unnecessarily critical or harsh when commenting on the author's work.

The Publication and Dissemination Unit gave me the opportunity to search for the theory of the publication process from different sources and put the obtained knowledge into practice. At the end of Part 1 of the course I felt confident to start the Professional Research and Development Project. I believe the first part of the course was very well planned and structured in a way that the researcher could start his/her journey armed with relevant skills needed to combat any challenge. That was how my journey started.

8.2. The professional research and development project

As a systematic process, the general purpose of research is to contribute to the body of knowledge that shapes and guides clinical practice settings (Tarling & Crofts, 2002). To be evidence-based and clinically useful, clinical practice must balance the strengths and limitations of all relevant research evidence with the practical realities of the healthcare and clinical settings. To classify a research report as strong or weak evidence in practice, it is necessary to evaluate the quality of the research as well as the reported outcome. It may not be appropriate simply to accept the conclusion

reported by the researchers, as additional factors need to be considered such as methodologies, instrumentations and interventions.

To find out about the accuracy of a diagnostic test, we need to perform a proper cross sectional study of patients with the relevant disorder. The ambitious aim of evidence based medicine and evidence based pathology in particular, is to advance clinical diagnosis of disease through research, and dissemination of new knowledge that meet high standards of critical review. If the current methodologies are not fit for purpose, improvement and employment of new techniques and advances should be constantly studied. Once a new technique becomes available, through research and innovation, then this should be thoroughly investigated for its suitability in clinical practice (Figure 8.1).

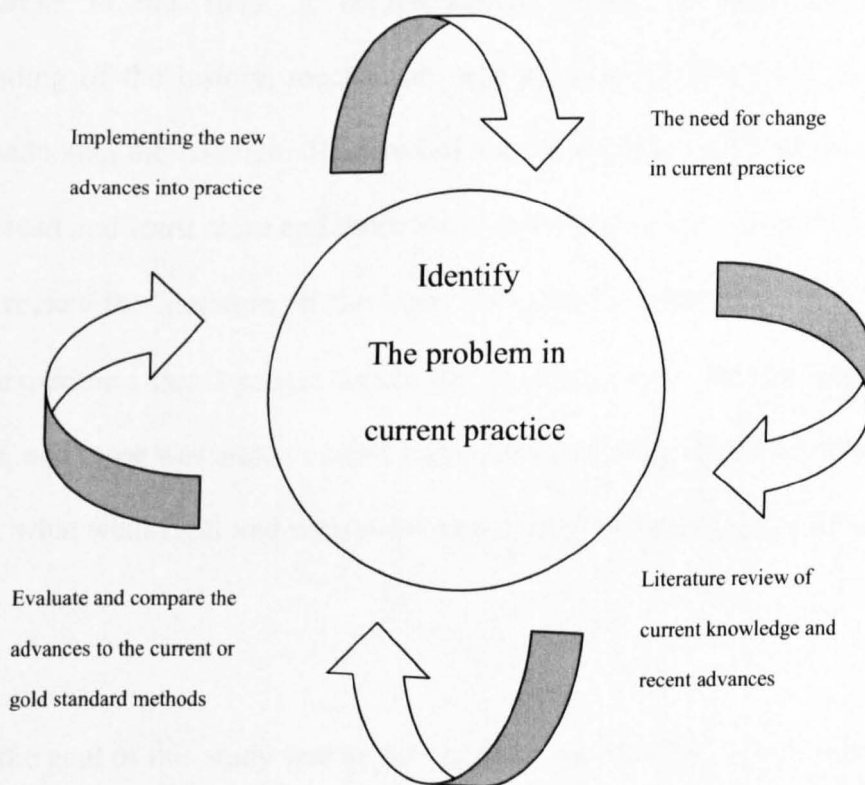


Figure 8.1. Reflection cycle adapted to clinical research

Identification of a problem in current practice and the cycle reviewing, evaluating and implementing new changed to improve the outcome.

Research questions within the context of evidence-based health care usually start in practice and can concern any aspect of the clinical encounter. The KDHCTU, where I work as a senior biomedical scientist, is one of the largest haemophilia treatment centres in Europe. Therefore the chosen topic of the research had to be in line with the objectives of the KDHCTU as a comprehensive care centre for patients with inherited and acquired bleeding disorders, including diagnosis and management. The topic of my thesis was to evaluate global assays in a group of patients with an inherited bleeding disorder, haemophilia; and the research hypothesis was that the 'global assays can predict the severity and clinical phenotype of patients with haemophilia better than the current conventional methods'.

Although the topic was broad and challenging, I was ready to embark on it to advance my expertise in this field. A comprehensive review of literature and a full understanding of the history, mechanism, and prognosis of the topic were needed before conducting the research. This created a great opportunity for me to refresh my memory, read and learn more and more about the whole process of haemostasis, and critically review the literature on the topic. The critical appraisal of literature was a valuable experience that I gained during the Publication and Dissemination Unit of the course, and more was added when I learned to reflect upon it and understand what happened, what went right and what went wrong, and why things turned out the way they did.

Although the goal of this study was to put literature into practice, it was always in my mind to bring novelty into the project to make it more fulfilling and rewarding. Therefore, I obliged myself to search even deeper in order to obtain the appropriate

knowledge to interpret and appraise the literature. This was not without challenges in an ever evolving field of science with a number of dedicated researchers around the world, working hard to find a better way of diagnosing, managing and, ultimately, caring for patients with haemophilia. This required keeping up-to-date with all publications on the topic, around the world and to find out what has been added to the current knowledge, any agreement or disagreement from previous understandings, and how my work could contribute to the current knowledge, at least at local level. This was particularly hard for a part-time student with a job and family commitments.

Once the research question was outlined, I started to work on the research proposal. With the kind help of my supervisors the proposal was written and an application for a research grant was sent to the Katharine Dormandy Trust. The ethical approval is a vital component of the research process. The process was long and the quantity of paperwork required was huge. Writing the patient's information sheet as a part the application process was interesting and thoughtful.

The optimisation and some modifications of methodology for the TGT and ROTEM[®] was next in the study protocol. At the time of the study there were only a few publications on the application of TGT and ROTEM[®] in diagnosis of individuals with haemophilia. The use of fluorogenic substrate was also limited to a few other studies. The use of a contact pathway inhibitor, the CTI, was just found to be useful in TGT in 2005, and the use of CTI in the ROTEM[®] analysis was evaluated for the first time in the study reported here and it was a great opportunity to publish the outcome. Another improvement in this study was a further spinning of the plasma samples in order to minimise the effect of microparticles and platelet remnant in the TGT. This was found

when I tested the double spun plasma samples for the presence of phospholipids in the samples by kaolin clotting time (KCT) test. The KCT is essentially an APTT test without added phospholipids. The double spun plasma samples had significantly shorter KCT than the triple spun plasma samples, therefore, the third spin, which was at 6000g for 2 minutes, was added to the study protocol.

At the beginning of the study, CAT was not yet in the market. Therefore, I performed the tests manually, in which the phospholipid vesicles had to be prepared by adding the precise amount of three different types of synthetic phospholipids in order to prepare a near physiologic substrate resembling platelet's surface. The tissue factor was readily available, but its concentration had to be optimised for the purpose of TGT.

Reflecting on the process of making these reagents, it gives me a sense of fulfilment in which I obtained experience in every stage of the TGT, from making and optimising the reagents to applying the test and make it work in a way which would compete with the CAT. Although, I did not invent any of these different processes, looking at different publications for different parts and gathering them all together to produce a protocol and then witnessing the outcome was a great experience.

By the end this experimental analysis, a test protocol for TGT and ROTEM[®] was produced which was unique at the time for three reasons:

- The use of CTI to inhibit the contact activation and mimic *in vivo* haemostasis as close as possible.

- The use of third spin to eliminate the interference of phospholipids in platelet poor plasma TGT.
- The use of freshly made reagents rather than commercially lyophilised reagents.

However, patient recruitment and sample collection was a long and time consuming process. The amount of work involved in this huge project, and the delay in the clinical data collections made it impossible to publish any paper in time before other publications in the same subject. We did, however, manage to present two posters at the International Society on Thrombosis and Haemostasis (ISTH) in 2007 and 2009, this was a huge privilege.

Patient recruitment was planned with the help of Dr Pratima Chowdary, the KDHC&TU Consultant Haematologist and Ms Patricia Lilly, Research Nurse. Due to the time restriction and long process of sample preparation, particularly for plasma rich platelet (PRP) samples, only two to three patients could be recruited each week. The TGT on PRP and ROTEM[®] analysis had to be performed on the same day and the rest was frozen for batch analysis.

Once the recruitment was completed and enough samples were collected, a plan for analysis was drawn up with the help of my laboratory supervisor, Miss Anne Riddell. It was a huge task to analyse all the samples for a large number of tests. Over 15,000 items of data were inserted into Excel spreadsheet for 97 patients and 22 normal individuals. Statistical analysis was a huge task and I had to learn to be able to perform statistical analysis. With the help of the KDHC&TU data manager and

statistician Mrs Anja Griffioen, I learnt to perform statistical analysis on the data. This was a great experience and another learning outcome for me arising from the project.

The next task was to obtain clinical information from patients' notes or from the individuals themselves. The process of going through medical notes and obtaining the clinical phenotype of patients was the most time consuming part of this research project. First of all, I could not perform the task myself because a clinician was needed to investigate the medical notes. Secondly, this part of the study was blinded therefore I could not go through the clinical notes, and thirdly, as a result of change of consultant haematologist, inevitably, the task was postponed until the new consultant took the responsibility for this aspect of the study. Reflecting on this part of the study, there was a period of time when I almost lost my inspiration. I struggled to find the motivation to continue when I thought that the research would fall through because of lack of support from the clinical team. However, the new consultant, Dr Pratima Chowdary, took responsibility and up until the submission of this thesis helped me to complete the task.

During the process of writing I have had to revise my thesis outline, and adjusted my statements of problem and research questions. This, no doubt came as a result of communication with my supervisors at work and the University, analysing data, and preparing several drafts of my thesis chapters. Once I finished writing the thesis, I found it necessary to consider the final shape of the document for submission. The University has detailed a policy and procedures for the presentation of a professional doctorate thesis which needs to be carefully studied and applied.

Some of the challenges I faced were compounded by factors related to the fact that I am a full-time employee and a part-time student. There were times when I felt overwhelmed by the amount of work I was faced with and struggled to see what was relevant. I experienced a rollercoaster ride of emotions, which cannot have been easy for those around me either, both at work and at home.

Supervision was an invaluable source of support and affirmation. When I struggled to understand, my supervisors, whether in the workplace or at the University, helped me to work things out; when I was frustrated with the speed at which things were moving, they allowed me to vent my frustrations; when I became fed up with looking at my research, they told me I was normal. I would advise anyone who plans to embark on a research project to have clear questions in mind and good support and supervision. These are invaluable.

The research journey was accompanied by a parallel journey of my own. It has been a satisfying, difficult, tedious, demanding, emotionally turbulent, intellectually stimulating, and ultimately transformative journey. I will never be the same again.

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Appendices

Appendix 1

APTT and FVIII/FIX activity levels as measured by one-stage factor assay.

<i>Mild HA</i>	<i>APTT</i>	<i>FVIII</i>	<i>Moderate</i>	<i>APTT</i>	<i>FVIII</i>	<i>Mild HB</i>	<i>APTT</i>	<i>FIX</i>	<i>Moderate</i>	<i>APTT</i>	<i>FIX</i>
	<i>s</i>	<i>IU/dL</i>	<i>HA</i>	<i>s</i>	<i>IU/dL</i>		<i>s</i>	<i>IU/dL</i>	<i>HB</i>	<i>s</i>	<i>IU/dL</i>
MiHA 1	44.0	27	MoHA 1	56.6	2	MiHB 1	49.6	6	MoHB 1	50.0	4
MiHA 2	46.5	8	MoHA 2	50.8	5	MiHB 2	41.6	7	MoHB 2	53.5	2
MiHA 3	39.0	15	MoHA 3	46.4	5	MiHB 3	45.3	6	MoHB 3	58.3	2
MiHA 4	42.2	10	MoHA 4	48.0	4	MiHB 4	39.4	13			
MiHA 5	48.1	8	MoHA 5	53.5	2						
MiHA 6	41.5	17	MoHA 6	50.1	3						
MiHA 7	46.3	10	MoHA 7	66.1	1						
MiHA 8	41.2	14	MoHA 8	52.7	5						
MiHA 9	75.9	11	MoHA 9	46.0	5						
MiHA 10	40.9	11	MoHA 10	45.0	3						
MiHA 11	40.1	14	MoHA 11	54.8	2						
MiHA 12	44.0	10									
MiHA 13	46.7	14									
MiHA 14	35.8	48									
MiHA 15	48.7	6									
MiHA 16	38.0	13									

MiHA = mild haemophilia A, MoHA = moderate haemophilia A,

MiHB = mild haemophilia B, MoHB = moderate haemophilia B.